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(57) Abstract

Analogues of the Kunitz Protease Inhibitor (KPI) domain of amyloid precursor protein bind to and inhibit activity of serine proteases, including kallikrein, plasmin and coagulation factors such as factors VIIa, IXa, Xa, XIa and XIIa. Pharmaceutical compositions containing the KPI analogues, along with methods for using such compositions, are useful for ameliorating and treating clinical conditions associated with increased serine protease activity, such as blood loss related to cardiopulmonary bypass surgery. Nucleic acid sequences encoding these analogues and systems for expression of the peptides of the invention are provided.

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KUNITZ TYPE PROTEASE INHIBITORS

Background of the Invention

The plasma, or serine, proteases of the blood contact system are known to be activated by interaction with negatively charged surfaces. For example, tissue injury during surgery exposes the vascular basement membrane, causing interaction of the blood with collagen, which is negatively charged at physiological pH. This induces a cascade of proteolytic events, leading to production of plasmin, a fibrinolytic protease, and consequent blood loss.

Perioperative blood loss of this type can be particularly severe during cardiopulmonary bypass (CPB) surgery, in which the patient's blood flow is diverted to an artificial heart-lung machine. CPB is an essential component of a number of life-saving surgical procedures. For example, in the United States, it is estimated that 300,000 patients every year undergo coronary artery bypass grafts involving the use of CPB.

Although necessary and generally safe, CPB is associated with a significant rate of morbidity, some of which may be attributed to a "whole body inflammatory response" caused by activation of plasma protease systems and blood cells through interactions with the artificial surfaces of the heart-lung machine (Butler *et al.*, *Ann. Thorac. Surg.* 55:552 (1993); Edmunds *et al.*, *J. Card. Surg.* 8:404 (1993)). For example, during extracorporeal circulation, exposure of blood to negatively charged surfaces of the artificial bypass circuit, e.g., plastic surfaces in the heart-lung machine, results in direct activation of plasma factor XII.

Factor XII is a single-chain 80 kDa protein that circulates in plasma as an inactive zymogen. Contact with negatively charged nonendothelial surfaces, like those of the bypass circuit, causes surface-bound factor XII to be autoactivated to the active serine protease factor XIIa. See Colman, *Agents Actions Suppl.* 42:125

(1993). Surface-activated factor XIIa then processes prekallikrein (PK) to active kallikrein, which in turn cleaves more XIIa from XII in a reciprocal activation reaction that results in a rapid amplification of the contact pathway. Factor XIIa can also activate the first component of complement C1, leading to production of the anaphylatoxin C5a through the classical complement pathway.

The CPB-induced inflammatory response includes changes in capillary permeability and interstitial fluid accumulation. Cleavage of high molecular weight kininogen (HK) by activated kallikrein generates the potent vasodilator bradykinin, which is thought to be responsible for increasing vascular permeability, resulting in edema, especially in the lung. The lung is particularly susceptible to damage associated with CPB, with some patients exhibiting what has been called "pump lung syndrome" following bypass, a condition indistinguishable from adult respiratory distress. See Johnson *et al.*, *J. Thorac. Cardiovasc. Surg.* 107:1193 (1994).

Post-CPB pulmonary injury includes tissue damage thought to be mediated by neutrophil sequestration and activation in the microvasculature of the lung. (Butler *et al.*, *supra*; Johnson, *et al.*, *supra*). Activated factor XII can itself stimulate neutrophil aggregation. Factor XIIa-generated kallikrein, and complement protein C5a generated by Factor XIIa activation of the complement cascade, both induce neutrophil chemotaxis, aggregation and degranulation. See Edmunds *et al.*, *supra* (1993). Activated neutrophils may damage tissue through release of oxygen-derived free-radicals, proteolytic enzymes such as elastase, and metabolites of arachidonic acid. Release of neutrophil products in the lung can cause changes in vascular tone, endothelial injury and loss of vascular integrity.

Intrinsic inhibition of the contact system occurs through inhibition of activated XIIa by C1-inhibitor (C1-INH). See Colman, *supra*. During CPB, this natural

inhibitory mechanism is overwhelmed by massive activation of plasma proteases and consumption of inhibitors. A potential therapeutic strategy for reducing post-bypass pulmonary injury mediated by neutrophil activation would, therefore, be to block the formation and activity of the neutrophil agonists kallikrein, factor XIIa, and C5a by inhibition of proteolytic activation of the contact system.

Protease inhibitor therapy which partially attenuates the contact system is currently employed clinically in CPB. Aprotinin, also known as basic pancreatic protease inhibitor (BPPI), is a small, basic, 58 amino acid polypeptide isolated from bovine lung. It is a broad spectrum serine protease inhibitor of the Kunitz type, and was first used during bypass in an attempt to reduce the inflammatory response to CPB. See Butler *et al.*, *supra*. Aprotinin treatment results in a significant reduction in blood loss following bypass, but does not appear to significantly reduce neutrophil activation. Additionally, since aprotinin is of bovine origin, there is concern that repeated administration to patients could lead to the development of an immune response to aprotinin in the patients, precluding its further use.

The proteases inhibited by aprotinin during CPB appear to include plasma kallikrein and plasmin. (See, e.g., Scott, *et al.*, *Blood* 69:1431 (1987)). Aprotinin is an inhibitor of plasmin (K_i of 0.23nM), and the observed reduction in blood loss may be due to inhibition of fibrinolysis through the blocking of plasmin action. Although aprotinin inhibits plasma kallikrein, (K_i of 20nM), it does not inhibit activated factor XII, and consequently only partially blocks the contact system during CPB.

Another attractive protease target for use of protease inhibitors, such as those of the present invention, is factor XIIa, situated at the very first step of contact activation. By inhibiting the proteolytic activity of factor XIIa, kallikrein production would be prevented, blocking amplification of

the contact system, neutrophil activation and bradykinin release. Inhibition of XIIa would also prevent complement activation and production of C5a. More complete inhibition of the contact system during CPB could, therefore, be achieved through the use of a better XIIa inhibitor.

Protein inhibitors of factor XIIa are known. For example, active site mutants of α_1-antitrypsin that inhibit factor XIIa have been shown to inhibit contact activation in human plasma. See Patston *et al.*, *J. Biol. Chem.* 265:10786 (1990). The large size and complexity (greater than 400 amino acid residues) of these proteins present a significant challenge for recombinant protein production, since large doses will almost certainly be required during CPB. For example, although it is a potent inhibitor of both kallikrein and plasmin, nearly 1 gram of aprotinin must be infused into a patient to inhibit the massive activation of the kallikrein-kinin and fibrinolytic systems during CPB.

The use of smaller, more potent XIIa inhibitors such as the corn and pumpkin trypsin inhibitors (Wen, *et al.*, *Protein Exp. & Purif.* 4:215 (1993); Pedersen, *et al.*, *J. Mol. Biol.* 236:385 (1994)) could be more cost-effective than the large α_1-antitrypsins, but the infusion of high doses of these non-mammalian inhibitors could result in immunologic reactions in patients undergoing repeat bypass operations. The ideal protein XIIa inhibitor is, therefore, preferably, small, potent, and of human sequence origin.

One candidate for an inhibitor of human origin is found in circulating isoforms of the human amyloid β-protein precursor (APPI), also known as protease nexin-2. APPI contains a Kunitz serine protease inhibitor domain known as KPI (Kunitz Protease Inhibitor). See Ponte *et al.*, *Nature*, 331:525 (1988); Tanzi *et al.*, *Nature* 331:528 (1988); Johnstone *et al.*, *Biochem. Biophys. Res. Commun.* 163:1248 (1989); Oltersdorf *et al.*, *Nature* 341:144 (1989). Human KPI shares about 45% amino acid sequence identity with aprotinin. The isolated KPI domain has

been prepared by recombinant expression in a variety of systems, and has been shown to be an active serine protease inhibitor. See, for example, Sinha, *et al.*, *J. Biol. Chem.* 265:8983 (1990). The measured *in vitro* K_i of KPI against plasma kallikrein is 45nM, compared to 20nM for aprotinin.

Aprotinin, KPI, and other Kunitz-type serine protease inhibitors have been engineered by site-directed mutagenesis to improve inhibitory activity or specificity. Thus, substitution of Lys^{15} of aprotinin with arginine resulted in an inhibitor with a K_i of 0.32nM toward plasma kallikrein, a 100-fold improvement over natural aprotinin. See PCT application No. 89/10374. See also Norris *et al.*, *Biol. Chem. Hoppe Seyler* 371:3742 (1990). Alternatively, substitution of position 15 of aprotinin with valine or substitution of position 13 of KPI with valine resulted in elastase inhibitors with K_is in the 100 pM range, although neither native aprotinin nor native KPI significantly inhibits elastase. See Wenzel *et al.*, in: *Chemistry of Peptides and Proteins, Vol. 3*, (Walter de Gruyter, Berlin, New York, 1986); Sinha *et al.*, *supra*. Methods for substituting residues 13, 15, 37, and 50 of KPI are shown in general terms in European Patent Application No. 0 393 431, but no specific sequences are disclosed, and no protease inhibition data are given.

Phage display methods have been recently used for preparing and screening derivatives of Kunitz-type protease inhibitors. See PCT Application No. 92/15605, which describes specific sequences for 34 derivatives of aprotinin, some of which were reportedly active as elastase and cathepsin inhibitors. The amino acid substitutions in the derivatives were distributed throughout almost all positions of the aprotinin molecule.

Phage display methods have also been used to generate KPI variants that inhibit factor VIIa and kallikrein. See Dennis *et al.*, *J. Biol. Chem.* 269:22129 and 269:22137 (1994). The residues that could be varied in the phage

display selection process were limited to positions 9-11, 13-17, 32, 36 and 37, and several of those residues were also held constant for each selection experiment. One of those variants was said to have a K_i of 1.2nM for kallikrein, and had substitutions at positions 9 (Thr→Pro), 13 (Arg→Lys), 15 (Met→Leu), and 37 (Gly→Tyr). None of the inhibitors was tested for the ability to inhibit factor XIIa.

It is apparent, therefore, that new protease inhibitors that can bind to and inhibit the activity of serine proteases are greatly to be desired. In particular it is highly desirable to prepare peptides, based on human peptide sequences, that can inhibit selected serine proteases such as kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. It is also highly desirable to prepare novel protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example by reducing pulmonary damage or blood loss during CPB.

Summary of the Invention

The present invention relates to peptides that can bind to and preferably exhibit inhibition of the activity of serine proteases. Those peptides can also provide a means of ameliorating, treating or preventing clinical conditions associated with increased activity of serine proteases. Particularly, the novel peptides of the present invention preferably exhibit a more potent and specific (i.e., greater) inhibitory effect toward serine proteases of interest in comparison to known serine protease inhibitors. Examples of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly

those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

In achieving the inhibition of serine protease activity, the invention provides protease inhibitors that can ameliorate one or more of the undesirable clinical manifestations associated with enhanced serine protease activity, for example, by reducing pulmonary damage or blood loss during CPB.

The present invention relates to protease inhibitors comprising the following amino acid sequences:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-X^3-Cys-Arg-Ala-X^4-X^5-X^6-X^7-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-X^8-Tyr-Gly-Gly-Cys-X^9-X^{10}-X^{11}-X^{12}-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein: X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr, Val, Ile and Ser; X^3 is selected from Pro and Ala; X^4 is selected from Arg, Ala, Leu, Gly, or Met; X^5 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^6 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^7 is selected from Arg, His, or Ala; X^8 is selected from Phe, Val, Leu, or Gly; X^9 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; X^{10} is selected from Ala, Arg, or Gly; X^{11} is selected from Lys, Ala, or Asn; and X^{12} is selected from Ser, Ala, or Arg.

The invention relates more specifically to protease inhibitors comprising the following amino acid sequences:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-X^3-Cys-Arg-Ala-X^4-X^5-X^6-X^7-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-X^8-Tyr-Gly-Gly-Cys-X^9-X^{10}-X^{11}-X^{12}-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr, Val, Ile and Ser; X^3 is selected from Pro and Ala; X^4 is selected from Arg, Ala, Leu, Gly, or Met; X^5 is selected from Ile, His, Leu, Lys,

Ala, or Phe; X^6 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^7 is selected from Arg, His, or Ala; X^8 is selected from Phe, Val, Leu, or Gly; X^9 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; X^{10} is selected from Ala, Arg, or Gly; X^{11} is selected from Lys, Ala, or Asn; X^{12} is selected from Ser, Ala, or Arg; provided that when X^4 is Arg, X^6 is Ile; when X^9 is Arg, X^4 is Ala or Leu; when X^9 is Tyr, X^4 is Ala or X^5 is His; and either X^5 is not Ile; or X^6 is not Ser; or X^9 is not Leu, Phe, Met, Tyr, or Asn; or X^{10} is not Gly; or X^{11} is not Asn; or X^{12} is not Arg.

Another aspect of this invention provides protease inhibitors wherein at least two amino acid residues selected from the group consisting of X^4, X^5, X^6, and X^7 defined above differ from the residues found in the naturally occurring sequence of KPI. Another aspect of this invention provides protease inhibitors wherein X^1 is Asp or Glu, X^2 is Thr, X^3 is Pro, and X^{12} is Ser. Yet another aspect of this invention provides protease inhibitors wherein X^1 is Glu, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Gly, and X^{11} is Asn. Another aspect of this invention provides protease inhibitors wherein X^1 is Asp, X^2 is Thr, X^3 is Pro, X^4 is Arg, X^5 is Ile, X^6 is Ile, X^7 is Arg, x^8 is Val, X^9 is Arg, X^{10} is Ala, and X^{11} is Lys. Another aspect of this invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Ala. Another aspect of this invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is Glu, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Ala, X^{11} is Asn, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is

Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Arg, X^{11} is Asn, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Val, Leu, or Gly, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Ala, x^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Pro, X^4 is Ala or Leu, X^5 is Ile, X^6 is Tyr, X^7 His, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg.

Yet another aspect of this invention provides protease inhibitors wherein X^2 is Thr, and X^4 is Ala. Another aspect of this invention provides protease inhibitors wherein X^2 is Thr, and X^4 is Leu. Another aspect of this invention provides protease inhibitors wherein X^2 is Val, and X^4 is Ala. Another aspect of this invention provides protease inhibitors wherein X^2 is Ser, and X^4 is Ala. Another aspect of this invention provides protease inhibitors wherein X^2 is Val, and X^4 is Leu. Another aspect of this invention provides protease inhibitors wherein X^2 is Ser, and X^4 is Leu.

Yet another aspect of this invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Leu, X^5 is Phe, X^6 is Lys, X^7 is Arg, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Leu, X^5 is Phe, X^6 is Lys, X^7 is Arg, X^8 is Phe, X^9 is Tyr, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg. Another aspect of this invention provides protease inhibitors wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Leu, X^5 is Phe, X^6 is Lys, X^7 is Arg, X^8 is Phe, X^9 is Leu, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg.

The present invention also relates to protease inhibitors comprising the following amino acid sequences:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-X^2-Ala-X^3-X^4-X^5-X^6-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein: X^1 is selected from Glu-Val-Val-Arg-Glu- and Asp-Val-Val-Arg-Glu-; X^2 is selected from Arg and Lys; X^3 is selected from Met, Arg, Ala, Leu, Ser, Val; X^4 is selected from Ile and Ala; X^5 is selected from Ser, Ile, Ala, Pro, Phe, Tyr, and Trp; and X^6 is selected from Arg, Ala, His, Gln, and Thr; provided that: when X^2 is Arg, X^3 is Leu, and X^4 is Ile, X^5 cannot be Ser; and also provided that either X^3 is not Met; or X^4 is not Ile; or X^5 is not Ser; or X^6 is not Arg. Another aspect of this invention provides protease inhibitors wherein X^3 is Arg or Met, and X^5 is Ser or Ile. Yet another aspect of this invention provides protease inhibitors wherein X^5 is selected from Phe, Tyr and Trp. Another aspect of this invention provides protease inhibitors wherein X^3 is Ala or Leu.

A further aspect of this invention provides an isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor of the invention. Another aspect of this invention provides an isolated DNA molecule comprising a DNA sequence encoding the protease inhibitor that further comprises an isolated DNA molecule operably linked to a regulatory sequence that controls expression of the coding sequence of the protease inhibitor in a host cell. Another aspect of this invention provides an isolated DNA molecule comprising a DNA sequence encoding the protease inhibitor operably linked to a regulatory sequence that controls expression of the coding sequence of the protease inhibitor in a host cell that further comprises a DNA sequence encoding a secretory signal peptide. That secretory signal peptide may preferably comprise the signal sequence of yeast alpha-mating

factor. Another aspect of this invention provides a host cell transformed with any of the DNA molecules defined above. Such a host cell may preferably comprise *E. coli* or a yeast cell. When such a host cell is a yeast cell, the yeast cell may be selected from *Saccharomyces cerevisiae* and *Pichia pastoris*.

Another aspect of this invention provides a method for producing a protease inhibitor of the present invention, comprising the steps of culturing a host cell as defined above and isolating and purifying said protease inhibitor.

A further aspect of this invention provides a pharmaceutical composition, comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle.

An additional aspect of this invention provides a method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle. That method of treatment may preferably be used to treat the clinical condition of blood loss during surgery.

Yet another aspect of this invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle.

Another aspect of this invention provides a method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition comprising a protease inhibitor of the present invention together with a pharmaceutically acceptable sterile vehicle, wherein said serine proteases

are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

A further aspect of this invention relates to protease inhibitors comprising the following amino acid sequences:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X^2-X^3-X^4-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X^5-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Ala, Leu, Gly, or Met; X^3 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^4 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^5 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr; provided that when X^5 is Arg, X^2 is Ala or Leu; when X^5 is Tyr, X^2 is Ala or X^3 is His; and either X^3 is not Ile; or X^4 is not Ser; or X^5 is not Leu, Phe, Met, Tyr, or Asn. Another aspect of this invention provides a protease inhibitor as defined above wherein X^1 is Glu, X^2 is Met, X^3 is Ile, X^4 is Ile, and X^5 is Gly.

The invention also relates more specifically to protease inhibitors comprising the following amino acid sequences:

Glu-Val-Val-Arg-Glu-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X^1-X^2-X^3-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X^4-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from Ala, Leu, Gly, or Met; X^2 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^3 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^4 is selected from Gly, Arg, Leu, Met, or Tyr; provided that when X^1 is Ala, X^2 is Ile, His, or

Leu; when X^1 is Leu, X^2 is Ile or His; when X^1 is Leu and X^2 is Ile, X^3 is not Ser; when X^1 is Gly, X^2 is Ile; when X^4 is Arg, X^1 is Ala or Leu; when X^4 is Tyr, X^1 is Ala or X^2 is His; and either X^1 is not Met, or X^2 is not Ile, or X^3 is not Ser, or X^4 is not Gly.

A further aspect of this invention provides a protease inhibitor as defined above wherein X^1 is Met, X^3 is Ser, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^2 is selected from His, Ala, Phe, Lys, and Leu. Another aspect of this invention provides a protease inhibitor wherein X^2 is His. Another aspect of this invention provides a protease inhibitor wherein X^2 is Ala. Another aspect of this invention provides a protease inhibitor wherein X^2 is Phe. Another aspect of this invention provides a protease inhibitor wherein X^2 is Lys. Another aspect of this invention provides a protease inhibitor wherein X^2 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^1 is Met, X^2 is Ile, and X^4 is Gly.

Yet another aspect of this invention provides a protease inhibitor wherein X^3 is Ile. Another aspect of this invention provides a protease inhibitor wherein X^3 is Pro. Another aspect of this invention provides a protease inhibitor wherein X^3 is Phe. Another aspect of this invention provides a protease inhibitor wherein X^3 is Tyr. Another aspect of this invention provides a protease inhibitor wherein X^3 is Trp. Another aspect of this invention provides a protease inhibitor wherein X^3 is Asn. Another aspect of this invention provides a protease inhibitor wherein X^3 is Leu.

An additional aspect of this invention provides a protease inhibitor wherein X^3 is Lys. Another aspect of this invention provides a protease inhibitor wherein X^3 is His. Another aspect of this invention provides a protease inhibitor wherein X^3 is Glu. Another aspect of this invention provides a protease inhibitor wherein X^1 is Ala. Another aspect of this invention provides a

protease inhibitor wherein X^2 is Ile. Another aspect of this invention provides a protease inhibitor wherein X^3 is Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^3 is Tyr, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^3 is Trp, and X^4 is Gly.

Yet another other aspect of this invention provides a protease inhibitor wherein X^3 is Ser or Phe, and X^4 is Arg or Tyr. Another aspect of this invention provides a protease inhibitor wherein X^2 is His or Leu, X^3 is Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^2 is His, X^3 is Asn or Phe, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^2 is Ile, X^3 is Pro, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Gly, X^2 is Ile, X^3 is Tyr, and X^4 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Met, X^2 is His, X^3 is Ser, and X^4 is Tyr.

Additionally, another aspect of this invention relates to protease inhibitors comprising the following amino acid sequences:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-Pro-Cys-Arg-Ala-X^3-X^4-X^5-X^6-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X^7-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu; X^2 is selected from Thr, Val, Ile and Ser; X^3 is selected from Arg, Ala, Leu, Gly, or Met; X^4 is selected from Ile, His, Leu, Lys, Ala, or Phe; X^5 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu; X^6 is selected from Arg, His, or Ala; and X^7 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr.

Another aspect of this invention provides a protease inhibitor as defined above wherein at least two amino acid residues selected from the group consisting of X^3,

X^4, X^5, and X^6 differ from the residues found in the naturally occurring sequence of KPI. Another aspect of this invention provides a protease inhibitor wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Ala or Leu, X^4 is Ile, X^5 is Tyr, X^6 is His and X^7 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^2 is Thr, and X^3 is Ala. Another aspect of this invention provides a protease inhibitor wherein X^2 is Thr, and X^3 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^2 is Val, and X^3 is Ala. Another aspect of this invention provides a protease inhibitor wherein X^2 is Ser, and X^3 is Ala. Another aspect of this invention provides a protease inhibitor wherein X^2 is Val, and X^3 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^2 is Ser, and X^3 is Leu. Another aspect of this invention provides a protease inhibitor wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Leu, X^4 is Phe, X^5 is Lys, X^6 is Arg and X^7 is Gly. Another aspect of this invention provides a protease inhibitor wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Leu, X^4 is Phe, X^5 is Lys, X^6 is Arg and X^7 is Tyr. Another aspect of this invention provides a protease inhibitor wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Leu, X^4 is Phe, X^5 is Lys, X^6 is Arg and X^7 is Leu.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

Brief Description of the Drawings

Figure 1 shows the strategy for the construction of plasmid pTW10:KPI.

Figure 2 shows the sequence of the synthetic gene for KPI (1→57) fused to the bacterial *phoA* secretory signal sequence.

Figure 3 shows the strategy for construction of plasmid pKPI-61.

Figure 4 shows the 192 bp *Xba*I-*Hin*dIII synthetic gene fragment encoding KPI (1→57) and four amino acids from yeast alpha-mating factor.

Figure 5 shows the synthetic 201 bp *Xba*I-*Hin*dIII fragment encoding KPI(-4→57) in PKPI-61.

Figure 6 shows the strategy for the construction of plasmid pTW113.

Figure 7 shows plasmid PTW113, encoding the 445 bp synthetic gene for yeast alpha-factor-KPI(-4→57) fusion.

Figure 8 shows the amino acid sequence for KPI (-4→57).

Figure 9 shows the strategy for constructing plasmid pTW6165.

Figure 10 shows plasmid, PTW6165, encoding the 445 bp synthetic gene for alpha-factor-KPI(-4→57; M15A, S17W) fusion.

Figure 11 shows the sequences of the annealed oligonucleotide pairs used to construct plasmids PTW6165, pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, and pTW6174.

Figure 12 shows the sequence of plasmid PTW6166 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15A, S17Y).

Figure 13 shows the sequence of plasmid PTW6175 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17F).

Figure 14 shows the sequence of plasmid PBG028 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17Y).

Figure 15 shows the sequence of plasmid PTW6183 encoding the fusion of yeast alpha-factor and KPI(-4→57; I16H, S17F).

Figure 16 shows the sequence of plasmid PTW6184 encoding the fusion of yeast alpha-factor and KPI(-4→57; I16H, S17Y).

Figure 17 shows the sequence of plasmid PTW6185 encoding the fusion of yeast alpha-factor and KPI(-4→57; I16H, S17W).

Figure 18 shows the sequence of plasmid PTW6173 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15A, I16H).

Figure 19 shows the sequence of plasmid PTW6174 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, I16H).

Figure 20 shows the amino acid sequence of KPI (-4→57; M15A, S17W).

Figure 21 shows the amino acid sequence of KPI (-4→57; M15A, S17Y).

Figure 22 shows the amino acid sequence of KPI (-4→57; M15L, S17F).

Figure 23 shows the amino acid sequence of KPI (-4→57; M15L, S17Y).

Figure 24 shows the amino acid sequence of KPI (-4→57; I16H, S17F).

Figure 25 shows the amino acid sequence of KPI (-4→57; I16H, S17Y).

Figure 26 shows the amino acid sequence of KPI (-4→57; I16H, S17W).

Figure 27 shows the amino acid sequence of KPI (-4→57; M15A, S17F).

Figure 28 shows the amino acid sequence of KPI (-4→57; M15A, I16H).

Figure 29 shows the amino acid sequence of KPI (-4→57; M15L, I16H).

Figure 30 shows the construction of plasmid pSP26:*Amp*:F1.

Figure 31 shows the construction of plasmid pgIII.

Figure 32 shows the construction of plasmid p*Pho*A:KPI:gIII.

Figure 33 shows the construction of plasmid pLG1.

Figure 34 shows the construction of plasmid pAL51.

Figure 35 shows the construction of plasmid pAL53.

Figure 36 shows the construction of plasmid PSP26:*Amp*:F1:*Pho*A:KPI:gIII.

Figure 37 shows the construction of plasmid pDW1 #14.

Figure 38 shows the coding region for the fusion of *pho*A-KPI (1→55)-geneIII.

Figure 39 shows the construction of plasmid PDW1 14-2.

Figure 40 shows the construction of KPI Library 16-19.

Figure 41 shows the expression unit encoded by the members of KPI Library 16-19.

Figure 42 shows the *pho*A-KPI(1→55)-geneIII region encoded by the most frequently occurring randomized KPI region.

Figure 43 shows the construction of pDD185 KPI (-4→57; M15A, S17F).

Figure 44 shows the sequence of alpha-factor fused to KPI (-4→57; M15A, S17F).

Figure 45 shows the inhibition constants (K_is) determined for purified KPI variants against the selected serine proteases kallikrein, factor Xa, and factor XIIa.

Figure 46 shows the inhibition constants (K_is) determined for KPI variants against kallikrein, plasmin, and factors Xa, XIa, and XIIa.

Figure 47 shows the post-surgical blood loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

Figure 48 shows the post-surgical hemoglobin loss in pigs in the presence (KPI) and absence (NS) of KPI 185-1 (M15A, S17F).

Figure 49 shows the oxygen tension in the presence and absence of KPI, before CPB, immediately after CPB, and at 60 and 180 minutes after the end of CPB.

Figure 50 summarizes the results shown in Figures 47-49.

Figure 51 shows the sequence of plasmid PTW6166 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15A, S17Y).

Figure 52 shows the sequence of plasmid PTW6175 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17F).

Figure 53 shows the sequence of plasmid PBG028 encoding the fusion of yeast alpha-factor and KPI(-4→57; M15L, S17Y).

Figure 54 shows the inhibition constants (K_is) determined for KPI variants against kallikrein, plasmin, and factor XIIa.

Detailed Description

The present invention provides peptides that can bind to and preferably inhibit the activity of serine proteases. These inhibitory peptides can also provide a means of ameliorating, treating or preventing clinical conditions associated with increased activity of serine proteases. The novel peptides of the present invention preferably exhibit a more potent and specific (i.e., greater) inhibitory effect toward serine proteases of interest than known serine protease inhibitors. Examples of such proteases include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

Peptides of the present invention may be used to reduce the tissue damage caused by activation of the proteases of the contact pathway of the blood during surgical procedures such as cardiopulmonary bypass (CPB). Inhibition of contact pathway proteases reduces the "whole body inflammatory response" that can accompany contact pathway activation, and that can lead to tissue damage, and possibly death. The peptides of the present invention may also be used in conjunction with surgical procedures to reduce activated serine protease-associated perioperative and postoperative blood loss. For instance, perioperative blood loss of this type may be

particularly severe during CPB surgery. Pharmaceutical compositions comprising the peptides of the present invention may be used in conjunction with surgery such as CPB; administration of such compositions may occur preoperatively, perioperatively or postoperatively. Examples of other clinical conditions associated with increased serine protease activity for which the peptides of the present invention may be used include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced protease release; deep vein thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of preferable uses of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The invention is based upon the novel substitution of amino acid residues in the peptide corresponding to the naturally occurring KPI protease inhibitor domain of human amyloid β-amyloid precursor protein (APPI). These substitutions produce peptides that can bind to serine proteases and preferably exhibit an inhibition of the activity of serine proteases. The peptides also preferably exhibit a more potent and specific serine protease inhibition than known serine protease inhibitors. In accordance with the invention, peptides are provided that may exhibit a more potent and specific inhibition of one or more serine proteases of interest, e.g., kallikrein, plasmin and factors Xa, XIa, XIIa, and XIIa.

The present invention also includes pharmaceutical compositions comprising an effective amount of at least one of the peptides of the invention, in combination with a pharmaceutically acceptable sterile vehicle, as described in REMINGTON'S PHARMACEUTICAL SCIENCES: DRUG RECEPTORS AND RECEPTOR THEORY, (18th ed.), Mack Publishing Co., Easton, PA (1990).

A. Selection of sequences of KPI variants

The sequence of KPI is shown in Table 1. Table 2 shows a comparison of this sequence with that of aprotinin, with which it shares about 45% sequence identity. The numbering convention for KPI shown in Table 1 and used hereinafter designates the first glutamic acid residue of KPI as residue 1. This corresponds to residue number 3 using the standard numbering convention for aprotinin.

The crystal structure for KPI complexed with trypsin has been determined. See Perona *et al.*, *J. Mol. Biol.* 230:919 (1993). The three-dimensional structure reveals two binding loops within KPI that contact the protease. The first loop extends from residue Thr^{9} to Ile^{16}, and the second loop extends from residue Phe^{32} to Gly^{37}. The two protease binding loops are joined through the disulfide bridge extending from Cys^{12} to Cys^{36}. KPI contains two other disulfide bridges, between Cys^{3} and Cys^{53}, and between Cys^{28} to Cys^{49}.

This structure was used as a guide to inform our strategy for making the amino acid residue substitutions that will be most likely to affect the protease inhibitory properties of KPI. Our examination of the structure indicated that certain amino acid residues, including residues 9, 11, 13-18, 32, and 37-40, appear to be of particular significance in determining the protease binding properties of the KPI peptide. In a preferred embodiment of the invention two or more of those KPI peptide residues are substituted; such substitutions preferably occurring among residues 9, 11, 13-18, 32, and 37-40. In particular, we found that those substituted peptides, including peptides comprising substitutions of at least two of the four residues at positions 15-18, may exhibit more potent and specific serine protease inhibition toward selected serine proteases of interest than exhibited by the natural KPI peptide domain. Such substituted peptides may further comprise one or more additional substitutions at residues 9, 11, 13, 14, 32 and 37-40; in particular, such peptides may further

comprise a substitution at positions 9 or 37, or an additional substitution at residue 13. In particular, the peptides of the present invention preferably exhibit a greater potency and specificity for inhibiting one or more serine proteases of interest (e.g., kallikrein, plasmin and factors VIIa, IXa, Xa, XIa, and XIIa) than the potency and specificity exhibited by native KPI or other known serine protease inhibitors. That greater potency and specificity may be manifested by the peptides of the present invention by exhibiting binding constants for serine proteases of interest that are less than the binding constants exhibited by native KPI, or other known serine protease inhibitors, for such proteases.

As an initial guide to informing the choices of amino acid substitution for preparation of KPI variants, the sequences and protease inhibitory activities of aprotinin and KPI are compared. Aprotinin is twice as potent as wild-type KPI with respect to inhibition of human plasma kallikrein, and is 100-fold more potent as an inhibitor of human plasmin. There are three amino acid differences between aprotinin and wild-type KPI in the first protease binding loop extending from residues 9 to 17. A series of KPI variants may then be created, using the methods detailed below, where the residues present in aprotinin at positions 13, 15 and 17 are substituted with the residues found in KPI. The effect of such substitutions upon KPI inhibition of plasma kallikrein and plasmin is then determined.

These results show that substitution of arginine at position 13 by lysine significantly reduces the activity of the resulting protein as an inhibitor of plasma kallikrein. Similarly, substituting positions 15 and 17 of KPI with the corresponding residues found in aprotinin also decreases potency of the KPI variants against kallikrein. Substitutions of aprotinin residues at positions 13 and 15, however, increase the potency of KPI toward plasmin. The single change of methionine to arginine at position 15 (designated M15R) decreases the K_i against plasmin more than 10-fold. The change of

serine to isoleucine at position 17 (S17I) decreases the potency of KPI toward plasmin.

It is observed that single-amino acid substitutions in the first protease binding loop are generally additive, that is, combinations of single amino-acid substitutions, each of which individually enhance the potency toward plasmin, result in variants with even higher potency. The substitution R13K results in a plasmin K_i of 12.3, and the further exchange of M15R results in a K_i that is reduced to 1.45.

It appears, therefore, from these results that combinations of favorable single amino acid substitutions can result in enhanced potency of KPI variants. It is further apparent that substitution in KPI with the residues found in the aprotinin first protease binding loop is not always useful. Although aprotinin is a more potent kallikrein inhibitor than KPI, none of the combinations of aprotinin residues in KPI improve kallikrein inhibition.

To further investigate substitutions that might usefully enhance protease inhibition, a series of single substitutions in KPI is prepared where charged residues in the first protease binding loop are systematically replaced with alanine. This is intended to determine whether substitutions at these sites affect potency toward plasma kallikrein, factor XIIa or plasmin.

It is found that replacement of arginine at position 13 (R13A) drastically reduces KPI inhibition of kallikrein, XIIa or plasmin. The replacement I16A, however, significantly increases the K_i towards both kallikrein and plasmin, suggesting that this amino acid position is critical to inhibition of these proteases. The S17A substitution has little effect. The substitution R18A has little effect upon plasmin inhibition, but significantly impacts inhibition of kallikrein and factor XIIa. These results suggest that substitutions at positions I16 and R18 have the potential to significantly alter the potency of KPI toward kallikrein or plasmin.

These results also suggest that substitutions at residues M15 and S17 could have major effects upon inhibition of kallikrein, XIIa or plasmin. To investigate this further, two sets of yeast expression plasmids are prepared, using the methods described in detail below, in which either M15 or S17 are replaced with all possible amino acids.

Yeast are transformed with these two sets of plasmids, and 100 individual colonies are picked at random from each transformation. Small cultures are grown from each of these colonies, and their conditioned broth is harvested and tested for kallikrein inhibiting activity. The plasmids from colonies yielding cultures expressing KPI variants more potent than wild-type KPI are isolated, and the KPI domain are sequenced. It is found that only four 4 substitutions at position 15: M15A,M15L,M15S,M15V; and 4 substitutions at position 17: S17P,S17F,S17Y and S17W, result in KPI variants with improved potency toward kallikrein.

Combinations of these position 15 and 17 mutants are then prepared to test if their effects on potency of protease inhibition are additive. Four of these double mutants ([M15A,S17Y], [M15A,S17W], [M15L,S17Y] and [M15L,S17F]) are substantially more potent toward kallikrein and factor XIIa than the single amino acid substitutions on which they are based.

The results of changing arginine at positions 18 for alanine also suggest that substitutions at position 18 could affect inhibition of kallikrein and factor XIIa. The KPI double variant M13A,S17W (named TW6165 below) is used to construct a series of variants where all possible amino acid substitutions other than Cys and Arg are placed at position 18. Of these variants, three ([M13A,S17W, R18H], [M13A,S17W, R18Q], and [M13A,S17W, R18T]) are found to exhibit enhanced inhibition of kallikrein and Factor XIIa.

The results described above relate to proteins having the N-terminal sequence EVVREVCS- *et seq.*, as found in KPI (-4→57). The present invention also relates, however

to proteins wherein the N-terminal sequence may be varied, preferably by substituting aspartic acid at the N-terminus in place of the glutamic acid (*i.e.* the N-terminal sequence is DVVREVCS-). Other N-terminal sequences that may be used will be apparent to the skilled artisan, including a sequence lacking the first four amino acids of KPI(-4→57), *i.e.* having the sequence EVCS-.

By way of example, and as set forth in greater detail below, the serine protease inhibitory properties of peptides of the present invention were measured for the serine proteases of interest — kallikrein, plasmin and factors Xa, XIa, and XIIa. Methodologies for measuring the inhibitory properties of the KPI variants of the present invention are known to those skilled in the art, e.g., by determining the inhibition constants of the variants toward serine proteases of interest, as described in Example 4, *infra*. Such studies measure the ability of the novel peptides of the present invention to bind to one or more serine proteases of interest and to preferably exhibit a greater potency and specificity for inhibiting one or more serine protease of interest than known serine protease inhibitors such as native KPI.

The ability of the peptides of the present invention to bind one or more serine proteases of interest, particularly the ability of the peptides to exhibit such greater potency and specificity toward serine proteases of interest, manifest the clinical and therapeutic applications of such peptides. The clinical and therapeutic efficacy of the peptides of the present invention can be assayed by *in vitro* and *in vivo* methodologies known to those skilled in the art, e.g., as described in Example 5, *infra*.

Table 1: SEQUENCE OF KPI:

```
  1                 10                  20                  30
V R E V C S E Q A E T G P C R A M I S R W Y F D V T E G K C A P

                  40                  50
F F Y G G C G G N R N N F D T E E Y C M A V C G S A I
```

Table 2: COMPARISON OF KPI AND APROTININ SEQUENCES:

```
          1         10        20 30     40        50
KPI:   VREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI
           | |  |||| | | |         | |  | ||||  ||||   | ||  || |
BPTI:  RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA
       1        10        20        30        40        50
```

B. Methods of producing KPI variants

The peptides of the present invention can be created by synthetic techniques or recombinant techniques which employ genomic or cDNA cloning methods.

1. Production by chemical synthesis

Peptides of the present invention can be routinely synthesized using solid phase or solution phase peptide synthesis. Methods of preparing relatively short peptides such as KPI by chemical synthesis are well known in the art. KPI variants could, for example be produced by solid-phase peptide synthesis techniques using commercially available equipment and reagents such as those available from Milligen (Bedford, MA) or Applied Biosystems-Perkin Elmer (Foster City, CA). Alternatively, segments of KPI variants could be prepared by solid-phase synthesis and linked together using segment condensation methods such as those described by Dawson *et al.*, *Science* 266:776 (1994). During chemical synthesis of the KPI variants, substitution of any amino acid is achieved simply by replacement of the residue that is to be substituted with a different amino acid monomer.

2. Production by recombinant DNA technology

(a) Preparation of genes encoding KPI variants

In a preferred embodiment of the invention, KPI variants are produced by recombinant DNA technology. This requires the preparation of genes encoding each KPI variant that is to be made. Suitable genes can be constructed by oligonucleotide synthesis using commercially available equipment, such as that provided by Milligen and Applied Biosystems, *supra*. The genes can be prepared by synthesizing the entire coding and non-coding strands, followed by annealing the two strands. Alternatively, the genes can be prepared by ligation of smaller synthetic oligonucleotides by methods well known in the art. Genes encoding KPI variants are produced by

varying the nucleotides introduced at any step of the synthesis to change the amino acid sequence encoded by the gene.

Preferably, however, KPI variants are made by site-directed mutagenesis of a gene encoding KPI. Methods of site-directed mutagenesis are well known in the art. See, for example, Ausubel *et al.*, (eds.) CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (Wiley Interscience, 1987); PROTEIN ENGINEERING (Oxender & Fox eds., A. Liss, Inc. 1987). These methods require the availability of a gene encoding KPI or a variant thereof, which can then be mutagenized by known methods to produce the desired KPI variants. In addition, linker-scanning and polymerase chain reaction ("PCR") mediated techniques can be used for purposes of mutagenesis. See PCR TECHNOLOGY (Erlich ed., Stockton Press 1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, vols. 1 & 2, *loc. cit.*

A gene encoding KPI can be obtained by cloning the naturally occurring gene, as described for example in U.S. Patents Nos. 5,223,482 and 5,187,153, which are hereby incorporated by reference in their entireties. In particular, see columns 6-9 of U.S. Patent No. 5,187,153. See also PCT Application No. 93/09233. In a preferred embodiment of the invention a synthetic gene encoding KPI is produced by chemical synthesis, as described above. The gene may encode the 57-amino acid KPI domain shown in Table 1, or it may also encode additional N-terminal amino acids from the APPI protein sequence, such as the four amino acid sequence (Glu-Val-Val-Arg, designated residues -4 to -1) immediately preceding the KPI domain in APPI.

Production of the gene by synthesis allows the codon usage of the KPI gene to be altered to introduce convenient restriction endonuclease recognition sites, without altering the sequence of the encoded peptide. In a preferred embodiment of the invention, the synthetic KPI gene contains restriction endonuclease recognition sites that facilitate excision of DNA cassettes from the KPI gene. These cassettes can be replaced with small

synthetic oligonucleotides encoding the desired changes in the KPI peptide sequence. See Ausubel, *supra*.

This method also allows the production of genes encoding KPI as a fusion peptide with one or more additional peptide or protein sequences. The DNA encoding these additional sequences is arranged in-frame with the sequence encoding KPI such that, upon translation of the gene, a fusion protein of KPI and the additional peptide or protein sequence is produced. Methods of making such fusion proteins are well known in the art. Examples of additional peptide sequences that can be encoded in the genes are secretory signal peptide sequences, such as bacterial leader sequences, for example *omp*A and *pho*A, that direct secretion of proteins to the bacterial periplasmic space. In a preferred embodiment of the invention, the additional peptide sequence is a yeast secretory signal sequence, such as α-mating factor, that directs secretion of the peptide when produced in yeast.

Additional genetic regulatory sequences can also be introduced into the synthetic gene that are operably linked to the coding sequence of the gene, thereby allowing synthesis of the protein encoded by the gene when the gene is introduced into a host cell. Examples of regulatory genetic sequences that can be introduced are: promoter and enhancer sequences and transcriptional and translational control sequences. Other regulatory sequences are well known in the art. See Ausubel *et al.*, *supra*, and Sambrook *et al.*, *supra*.

Sequences encoding other fusion proteins and genetic elements are well known to those of skill in the art. In a preferred embodiment of the invention, the KPI sequence is prepared by ligating together synthetic oligonucleotides to produce a gene encoding an in-frame fusion protein of yeast α-mating factor with either KPI (1→57) or KPI (-4→57).

The gene constructs prepared as described above are conveniently manipulated in host cells using methods of manipulating recombinant DNA techniques that are well

known in the art. See, for example Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989), and Ausubel, *supra*. In a preferred embodiment of the invention the host cell used for manipulating the KPI constructs is *E. coli*. For example, the construct can be ligated into a cloning vector and propagated in *E. coli* by methods that are well known in the art. Suitable cloning vectors are described in Sambrook, *supra*, or are commercially available from suppliers such as Promega (Madison, WI), Stratagene (San Diego, CA) and Life Technologies (Gaithersburg, MD).

Once a gene construct encoding KPI has been obtained, genes encoding KPI variants are obtained by manipulating the coding sequence of the construct by standard methods of site-directed mutagenesis, such as excision and replacement of small DNA cassettes, as described *supra*. See Ausubel, *supra*, and Sinha *et al.*, *supra*. See also U.S. Patent 5,373,090, which is herein incorporated by reference in its entirety. See particularly, columns 4-12 of U.S. Patent 5,272,090. These genes are then used to produce the KPI variant peptides as described below.

Alternatively, KPI variants can be produced using phage display methods. See, for example, Dennis *et al.* *supra*, which is hereby incorporated by reference in its entirety. See also U.S. Patent Nos. 5,223,409 and 5,403,484, which are hereby also incorporated by reference in their entireties. In these methods, libraries of genes encoding variants of KPI are fused in-frame to genes encoding surface proteins of filamentous phage, and the resulting peptides are expressed (displayed) on the surface of the phage. The phage are then screened for the ability to bind, under appropriate conditions, to serine proteases of interest immobilized on a solid support. Large libraries of phage can be used, allowing simultaneous screening of the binding properties of a large number of KPI variants. Phage that have desirable binding properties are isolated and the sequences of the genes encoding the corresponding KPI

variants is determined. These genes are then used to produce the KPI variant peptides as described below.

(b) Expression of KPI variant peptides

Once genes encoding KPI variants have been prepared, they are inserted into an expression vector and used to produce the recombinant peptide. Suitable expression vectors and corresponding methods of expressing recombinant proteins and peptides are well known in the art. Methods of expressing KPI peptides are described in U.S. Patent 5,187,153, columns 9-11, U.S. Patent 5,223,482, columns 9-11, and PCT application 93/09233, pp. 49-67. See also Ausubel *et al.*, *supra*, and Sambrook *et al.*, *supra*. The gene can be expressed in any number of different recombinant DNA expression systems to generate large amounts of the KPI variant, which can then be purified and tested for its ability to bind to and inhibit serine proteases of interest.

Examples of expression systems known to the skilled practitioner in the art include bacteria such as *E. coli*, yeast such as *Saccharomyces cerevisiae* and *Pichia pastoris*, baculovirus, and mammalian expression systems such as in Cos or CHO cells. In a preferred embodiment, KPI variants are expressed in *Pichia pastoris*. In another preferred embodiment the KPI variants are cloned into expression vectors to produce a chimeric gene encoding a fusion protein of the KPI variant with yeast α-mating factor. The mating factor acts as a signal sequence to direct secretion of the fusion protein from the yeast cell, and is then cleaved from the fusion protein by a membrane-bound protease during the secretion process. The expression vector is transformed into *S. cerevisiae*, the transformed yeast cells are cultured by standard methods, and the KPI variant is purified from the yeast growth medium.

Recombinant bacterial cells expressing the peptides of the present invention, for example, *E. coli*, are grown in any of a number of suitable media, for example LB, and the expression of the recombinant antigen induced by

adding IPTG to the media or switching incubation to a higher temperature. After culturing the bacteria for a further period of between 2 and 24 hours, the cells are collected by centrifugation and washed to remove residual media. The bacterial cells are then lysed, for example, by disruption in a cell homogenizer and centrifuged to separate dense inclusion bodies and cell membranes from the soluble cell components. This centrifugation can be performed under conditions whereby dense inclusion bodies are selectively enriched by incorporation of sugars such as sucrose into the buffer and centrifugation at a selective speed. If the recombinant peptide is expressed in inclusion bodies, as is the case in many instances, these can be washed in any of several solutions to assist in the removal of any contaminating host proteins, then solubilized in solutions containing high concentrations of urea (e.g., 8M) or chaotropic agents such as guanidine hydrochloride in the presence of reducing agents such as β-mercaptoethanol or DTT (dithiothreitol).

At this stage it may be advantageous to incubate the peptides of the present invention for several hours under conditions suitable for the peptides to undergo a refolding process into a conformation which more closely resembles that of native KPI. Such conditions generally include low protein concentrations less than 500 μg/ml, low levels of reducing agent, concentrations of urea less than 2M and often the presence of reagents such as a mixture of reduced and oxidized glutathione which facilitate the interchange of disulphide bonds within the protein molecule. The refolding process can be monitored, for example, by SDS-PAGE or with antibodies which are specific for the native molecule (which can be obtained from animals vaccinated with the native molecule isolated from parasites). Following refolding, the peptide can then be purified further and separated from the refolding mixture by chromatography on any of several supports including ion exchange resins, gel permeation resins or on a variety of affinity columns.

Purification of KPI variants can be achieved by standard methods of protein purification, e.g., using various chromatographic methods including high performance liquid chromatography and adsorption chromatography. The purity and the quality of the peptides can be confirmed by amino acid analyses, molecular weight determination, sequence determination and mass spectrometry. See, for example, PROTEIN PURIFICATION METHODS — A PRACTICAL APPROACH, Harris *et al.*, eds. (IRL Press, Oxford, 1989). In a preferred embodiment, the yeast cells are removed from the growth medium by filtration or centrifugation, and the KPI variant is purified by affinity chromatography on a column of trypsin-agarose, followed by reversed-phase HPLC.

C. Measurement of protease inhibitory properties of KPI variants

Once KPI variants have been purified, they are tested for their ability to bind to and inhibit serine proteases of interest *in vitro*. The peptides of the present invention preferably exhibit a more potent and specific inhibition of serine proteases of interest than known serine protease inhibitors, such as the natural KPI peptide domain. Such binding and inhibition can be assayed for by determining the inhibition constants for the peptides of the present invention toward serine proteases of interest and comparing those constants with constants determined for known serine protease inhibitors, e.g., the native KPI domain, toward those proteases. Methods for determining inhibition constants of protease inhibitors are well known in the art. See Fersht, ENZYME STRUCTURE AND MECHANISM, 2nd ed., W.H. Freeman and Co., New York, (1985).

In a preferred embodiment the inhibition experiments are carried out using a chromogenic synthetic protease substrate, as described, for example, in Bender *et al.*, *J. Amer. Chem. Soc.* 88:5890 (1966). Measurements taken by this method can be used to calculate inhibition

constants (K_i values) of the peptides of the present invention toward serine proteases of interest. See Bieth in BAYER-SYMPOSIUM V "PROTEINASE INHIBITORS", Fritz *et al.*, eds., pp. 463-69, Springer-Verlag, Berlin, Heidelberg, New York, (1974). KPI variants that exhibit potent and specific inhibition of one or more serine proteases of interest may subsequently be tested *in vivo*. *In vitro* testing, however, is not a prerequisite for *in vivo* studies of the peptides of the present invention.

D. Testing of KPI variants *in vivo*

The peptides of the present invention may be tested, alone or in combination, for their therapeutic efficacy by various *in vivo* methodologies known to those skilled in the art, e.g., the ability of KPI variants to reduce postoperative bleeding can be tested in standard animal models. For example, cardiopulmonary bypass surgery can be carried out on animals such as pigs in the presence of KPI variants, or in control animals where the KPI variant is not used. The use of pigs as a model for studying the clinical effects associated with CPB has previously been described. See Redmond *et al.*, *Ann. Thorac. Surg.* 56:474 (1993).

The KPI variant is supplied to the animals in a pharmaceutical sterile vehicle by methods known in the art, for example by continuous intravenous infusion. Chest tubes can be used to collect shed blood for a defined period of time. The shed blood, together with the residual intrathoracic blood found after sacrifice of the animal can be used to calculate hemoglobin (Hgb) loss. The postoperative blood and Hgb loss is then compared between the test and control animals to determine the effect of the KPI variants.

E. Therapeutic use of KPI variants

KPI variants of the present invention found to exhibit therapeutic efficacy (e.g., reduction of blood loss following surgery in animal models) may preferably be used and administered, alone or in combination or as

a fusion protein, in a manner analogous to that currently used for aprotinin or other known serine protease inhibitors. See Butler *et al., supra*. Peptides of the present invention generally may be administered in the manner that natural peptides are administered. A therapeutically effective dose of the peptides of the present invention preferably affects the activity of the serine proteases of interest such that the clinical condition may be treated, ameliorated or prevented. Therapeutically effective dosages of the peptides of the present invention can be determined by those skilled in the art, e.g., through *in vivo* or *in vitro* models. Generally, the peptides of the present invention may be administered in total amounts of approximately 0.01 to approximately 500, specifically 0.1 to 100 mg/kg body weight, if desired in the form of one or more administrations, to achieve therapeutic effect. It may, however, be necessary to deviate from such administration amounts, in particular depending on the nature and body weight of the individual to be treated, the nature of the medical condition to be treated, the type of preparation and the administration of the peptide, and the time interval over which such administration occurs. Thus, it may in some cases be sufficient to use less than the above amount of the peptides of the present invention, while in other cases the above amount is preferably exceeded. The optimal dose required in each case and the type of administration of the peptides of the present invention can be determined by one skilled in the art in view of the circumstances surrounding such administration. Such peptides can be administered by intravenous injections, *in situ* injections, local applications, inhalation, oral administration using coated polymers, dermal patches or other appropriate means. Compositions comprising peptides of the present invention are advantageously administered in the form of injectable compositions. Such peptides may be preferably administered to patients via continuous intravenous infusion, but can also be administered by single or

multiple injections. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described in REMINGTON'S PHARMACEUTICAL SCIENCES, pp. 1405-12 and 1461-87 (1975) and THE NATIONAL FORMULARY XIV., 14th Ed. Washington: American Pharmaceutical Association (1975). Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobials, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components of the composition are adjusted according to routine skills in the art. See GOODMAN AND GILMAN'S THE PHARMACOLOGICAL BASIS FOR THERAPEUTICS (7th ed.). The peptides of the present invention may be present in such pharmaceutical preparations in a concentration of approximately 0.1 to 99.5% by weight, specifically 0.5 to 95% by weight, relative to the total mixture. Such pharmaceutical preparations may also comprise other pharmaceutically active substances in addition to the peptides of the present invention. Other methods of delivering the peptides to patients will be readily apparent to the skilled artisan.

Examples of mammalian serine proteases that may exhibit inhibition by the peptides of the present invention include: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as thrombin and factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator. Examples of conditions associated with increased serine protease activity include: CPB-induced inflammatory response; post-CPB pulmonary injury; pancreatitis; allergy-induced

protease release; deep vein thrombosis; thrombocytopenia; rheumatoid arthritis; adult respiratory distress syndrome; chronic inflammatory bowel disease; psoriasis; hyperfibrinolytic hemorrhage; organ preservation; wound healing; and myocardial infarction. Other examples of the use of the peptides of the present invention are described in U.S. Patent No. 5,187,153.

The inhibitors of the present invention may also be used for inhibition of serine protease activity *in vitro*, for example during the preparation of cellular extracts to prevent degradation of cellular proteins. For this purpose the inhibitors of the present invention may preferably be used in a manner analogous to the way that aprotinin, or other known serine protease inhibitors, are used. The use of aprotinin as a protease inhibitor for preparation of cellular extracts is well known in the art, and aprotinin is sold commercially for this purpose.

The present invention, thus generally described, will be understood more readily by reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

EXAMPLES

Example 1. Expression of wild-type KPI (-4→57)

A. Construction of PTW10:KPI

Plasmid PTW10:KPI is a bacterial expression vector encoding the 57 amino acid form of KPI fused to the bacterial *pho*A signal sequence. The strategy for the construction of PTW10:KPI is shown in Figure 1.

Plasmid pcDNAII (Invitrogen, San Diego, CA) was digested with *Pvu*II and the larger of the two resulting *Pvu*II fragments (3013 bp) was isolated. Bacterial expression plasmid pSP26 was digested with *Mlu*I and *Rsr*II, and the 409 bp *Mlu*I-*Rsr*II fragment containing the pTrp promoter element and transcription termination signals was isolated by electrophoresis in a 3% NuSieve Agarose gel (FMC Corp., Rockland, ME). Plasmid pSP26, containing a heparin-binding EGF-like growth factor (HB-EGF) insert between the *Nde*I and *Hind*III sites, is

described as pNA28 in Thompson *et al.*, *J. Biol. Chem.* 269:2541 (1994). Plasmid pSP26 was deposited in host *E. coli* W3110, pSP26 with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, 20852, USA under the conditions specified by the Budapest Treaty on the International Recognition of the Deposit of Microorganisms (Budapest Treaty). Host *E. coli* W3110, pSP26 was deposited on 3 May 1995 and given Accession No. 69800. Availability of the deposited plasmid is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The ends of the *Mlu*I-*Rsr*II fragment were blunted using DNA polymerase Klenow fragment by standard techniques. The blunted fragment of pSP26 was then ligated into the large *Pvu*II fragment of plasmid pCDNAII, and the ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and used to isolate plasmid pTW10 by standard techniques.

A synthetic gene was constructed encoding the bacterial *phoA* secretory signal sequence fused to the amino terminus of KPI(1→57). The synthetic gene contains cohesive ends for *Nde*I and *Hin*dIII, and also incorporates restriction endonuclease recognition sites for *Age*I, *Rsr*II, *Aat*II and *Bam*HI, as shown in Figure 2. The synthetic *pho*A-KPI gene was constructed from 6 oligonucleotides of the following sequences (shown 5'→3'):

6167:
TATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCC
CTGTGACAAAAGCCGAGGTGTGCTCTGAA

6169:
CTCGGCTTTTGTCACAGGGGTAAACAGTAACGGTAAGAGTGCCAGTGCAA
TAGTGCTTTGTTTCATA

6165:
CAAGCTGAGACCGGTCCGTGCCGTGCAATGATCTCCCGCTGGTACTTTGA
CGTCACTGAAGGTAAGTGCGCTCCATTCTTT

6166:
GCACTTACCTTCAGTGACGTCAAAGTACCAGCGGGAGATCATTGCACGGC
ACGGACCGGTCTCAGCTTGTTCAGAGCACAC

6168:
TACGGCGGTTGCGGCGGCAACCGTAACAACTTTGACACTGAAGAGTACTG
CATGGCAGTGTGCGGATCCGCTATTTAAGCT

6164:
AGCTTAAATAGCGGATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAA
AGTTGTTACGGTTGCCGCCGCAACCGCCGTAAAAGAATGGAGC

The oligonucleotides were phosphorylated and annealed in pairs: 6167 + 6169, 6165 + 6166, 6168 + 6164. In 20 μl T4 DNA Ligase Buffer (New England Biolabs, Beverley, MA), 1 μg of each oligonucleotide pair was incubated with 10 U T4 Polynucleotide Kinase (New England Biolabs) for 1 h at 37°C, then heated to 95°C for 1 minute, and slow-cooled to room temperature to allow annealing. All three annealed oligo pairs were then mixed for ligation to one another in a total volume of 100 μl T4 DNA Ligase Buffer, and incubated with 400 U T4 DNA Ligase (New England Biolabs) overnight at 15°C. The ligation mixture was extracted with an equal volume of phenol:$CHCl_3$ (1:1), ethanol-precipitated, resuspended in 50 μl Restriction Endonuclease Buffer #4 (New England Biolabs) and digested with *Nde*I and *Hin*dIII. The annealed, ligated and digested oligos were then subjected to electrophoresis in a 3% NuSieve Agarose gel, and the 240 bp *Nde*I-*Hin*dIII fragment was excised. This gel-purified synthetic gene was ligated into plasmid pTW10 which had previously been digested with *Nde*I and *Hin*dIII, and the ligation mixture was used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were selected and used to prepare plasmid pTW10:KPI. This plasmid contains the *pho*A-KPI(1→57) fusion protein inserted between the pTrp promoter element and the transcription termination signals.

B. Construction of pKPI-61

The strategy for constructing pKPI-61 is shown in Figure 3. Plasmid pTW10:KPI was digested with *Age*I and

*Hin*dIII; the resulting 152 bp *Age*I-*Hin*dIII fragment containing a portion of the KPI synthetic gene was isolated by preparative gel electrophoresis. An oligonucleotide pair (129 + 130) encoding the 9 amino-terminal residues of KPI(1→57) and 4 amino acids of yeast α-mating factor was phosphorylated and annealed as described above.

129: CTAGATAAAAGAGAGGTGTGCTCTGAACAAGCTGAGA
130: CCGGTCTCAGCTTGTTCAGAGCACACCTCTCTTTTAT

The annealed oligonucleotides were then ligated to the *Age*I-*Hin*dIII fragment of the KPI (1→57) synthetic gene. The resulting 192 bp *Xba*I-*Hin*dIII synthetic gene (shown in Figure 4) was purified by preparative gel electrophoresis, and ligated into plasmid pUC19 which had previously been digested with *Xba*I and *Hin*dIII. The ligation products were used to transform *E. coli* strain MC1061. Ampicillin-resistant colonies were picked and used to prepare plasmid PKPI-57 by standard methods. To create a synthetic gene encoding KPI(-4→57), PKPI-57 was digested with *Xba*I and *Age*I and the smaller fragment replaced with annealed oligos 234 + 235, which encode 4 amino acid residues of yeast α-mating factor fused a 4 amino acid residue amino-terminal extension of KPI(1→57).

234: CTAGATAAAAGAGAGGTTGTTAGAGAGGTGTGCTCTGAACAAGCTGAGA
235: CCGGTCTCAGCTTGTTCAGAGCACACCTCTCTAACAACCTCTCTTTTAT

The 4 extra amino acids are encoded in the amyloid β-protein precursor/protease nexin-2 (APPI) which contains the KPI domain. The synthetic 201 bp *Xba*I-*Hin*dIII fragment encoding KPI(-4→57) in pKPI-61 is shown in Figure 5.

C. Assembly of pTW113

The strategy for the construction of PTW113 is shown in Figure 6. Plasmid pSP35 was constructed from yeast expression plasmid pYES2 (Invitrogen, San Diego, CA) as

follows. A 267 bp *Pvu*II-*Xba*I fragment was generated by PCR from yeast α-mating factor DNA using oligos 6274 and 6273:

6274: GGGGGCAGCTGTATAAACGATTAAAA
6273: GGGGGTCTAGAGATACCCCTTCTTCTTTAG

This PCR fragment, encoding an 82 amino acid portion of yeast α-mating factor, including the secretory signal peptide and pro-region, was inserted into pYES2 that had been previously digested with *Pvu*II and *Xba*I. The resulting plasmid is denoted pSP34.

Two oligonucleotide pairs, 6294 + 6292 were then ligated to 6290 + 6291, and the resulting 135 bp fragment was purified by gel electrophoresis.

6294: CTAGATAAAAGAGAGGCTGAGGCTCACGCTGAAGGTACTTTCACTTC

6290: TGACGTCTCTTCTTACTTGGAAGGTCAAGCTGCTAAGGAATTCAT CGCTTGGTTGGTCAAAGGTAGAGGTTAAGCTTA

6291: CTAGTAAGCTTAACCTCTACCTTTGACCAACCAAGCGATGAATTC CTTAGCA

6292: GCTTGACCTTCCAAGTAAGAAGAGACGTCAGAAGTGAAAGTACCT TCAGCGTGAGCCTCAGCCTCTCTTTTAT

The resulting synthetic fragment was ligated into the *Xba*I site of pSP34, resulting in plasmid pSP35. pSP35 was digested with *Xba*I and *Hin*dIII to remove the insert, and ligated with the 201 bp *Xba*I-*Hin*dIII fragment of pKPI-61, encoding KPI(-4→57). The resulting plasmid pTW113, encodes the 445 bp synthetic gene for the α-factor-KPI(-4→57) fusion. See Figure 7.

D. Transformation of yeast with pTW113

Saccharomyces cerevisiae strain ABL115 was transformed with plasmid pTW113 by electroporation by the method of Becker *et al.*, *Methods Enzymol.* 194:182 (1991). An overnight culture of yeast strain ABL115 was used to inoculate 200 ml YPD medium. The inoculated culture was grown with vigorous shaking at 30°C to an OD_{600} of 1.3-1.5,

at which time the cells were harvested by centrifugation at 5000 rpm for 5 minutes. The cell pellet was resuspended in 200 ml ice-cold water, respun, resuspended in 100 ml ice-cold water, then pelleted again. The washed cell pellet was resuspended in 10 ml ice-cold 1M sorbitol, recentrifuged, then resuspended in a final volume of 0.2 ml ice-cold 1M sorbitol. A 40 μl aliquot of cells was placed into the chamber of a cold 0.2 cm electroporation cuvette (Invitrogen), along with 100 ng plasmid DNA for pTW113. The cuvette was placed into an Invitrogen Electroporator II and pulsed at 1500 V, 25 μF, 100 Ω. Electroporated cells were diluted with 0.5 ml 1M sorbitol, and 0.25 ml was spread on an SD agar plate containing 1M sorbitol. After 3 days' growth at 30°C, individual colonies were streaked on SD + CAA agar plates.

E. Induction of pTW113/ABL115, purification of KPI(-4→57)

Yeast cultures were grown in a rich broth and the galactose promoter of the KPI expression vector induced with the addition of galactose as described by Sherman, *Methods Enzymol.* 194:3 (1991). A single well-isolated colony of pTW113/ABL115 was used to inoculate a 10 ml overnight culture in Yeast Batch Medium. The next day, 1L Yeast Batch Medium which had been made 0.2% glucose was inoculated to an OD_{600} of 0.1 with the overnight culture. Following 24 hours at 30°C with vigorous shaking, the 1L culture was induced by the addition of 20 ml Yeast Galactose Feed Medium. Following induction, the culture was fed every 12 hours with the addition of 20 ml Yeast Galactose Feed Medium. At 48 hours after induction, the yeast broth was harvested by centrifugation, then adjusted to pH 7.0 with 2M Tris, pH 10. The broth was subjected to trypsin-Sepharose affinity chromatography, and bound KPI(-4→57) was eluted with 20mM Tris pH 2.5. See Schilling *et al.*, *Gene* 98:225 (1991). Final purification of KPI(-4→57) was accomplished by HPLC chromatography on a semi-prep Vydac

C4 column in a gradient of 20% to 35% acetonitrile. The sample was dried and resuspended in PBS at 1-2 mg/ml. The amino acid sequence of KPI(-4→57) is shown in Figure 8.

Example 2. Recombinant Expression of site-directed KPI(-4→57) variants

Expression vectors for the production of specific variants of KPI(-4→57) were all constructed using the pTW113 backbone as a starting point. For each KPI variant, an expression construct was created by replacing the 40 bp *Rsr*II-*Aat*II fragment of the synthetic KPI gene contained in pTW113 with a pair of annealed oligonucleotides which encode specific codons mutated from the wild-type KPI(-4→57) sequence. In the following Examples the convention used for designating the amino substituents in the KPI variants indicates first the single letter code for the amino acid found in wild-type KPI, followed by the position of the residue using the numbering convention described *supra*, followed by the code for the replacement amino acid. Thus, for example, M15R indicates that the methionine residue at position 15 is replaced by an arginine.

A. Construction of pTW6165

The strategy for constructing pTW6165 is shown in Figure 9. Plasmid pTW113 was digested with *Rsr*II and *Aat*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (812 + 813) was phosphorylated, annealed and gel-purified as described above.

812: GTCCGTGCCGTGCAGCTATCTGGCGCTGGTACTTTGACGT
813: CAAAGTACCAGCGCCAGATAGCTGCACGGCACG

The annealed oligonucleotides were ligated into the *Rsr*II and *Aat*II-digested pTW113, and the ligation product was used to transform *E. coli* strain MC1061. Transformed colonies were selected by ampicillin resistance. The

resulting plasmid, pTW6165, encodes the 445 bp synthetic gene for the α-factor-KPI(-4→57; M15A, S17W) fusion. See Figure 10.

B. Construction of pTW6166, pTW6175, pBG028, pTW6183, pTW6184, pTW6185, pTW6173, pTW6174.

Construction of the following KPI(-4→57) variants was accomplished exactly as outlined for pTW6165. The oligonucleotides utilized for each construct are denoted below, and the sequences of annealed oligonucleotide pairs are shown in Figure 11. Figures 12-19 show the synthetic genes for the α-factor fusions with each KPI(-4→57) variant.

pTW6166: KPI(-4→57; M15A, S17Y) — See Figure 12

814: GTCCGTGCCGTGCAGCTATCTACCGCTGGTACTTTGACGT

815: CAAAGTACCAGCGGTAGATAGCTGCACGGCACG

pTW6175: KPI(-4→57; M15L, S17F) — See Figure 13

867: GTCCGTGCCGTGCATTGATCTTCCGCTGGTACTTTGACGT

868: CAAAGTACCAGCGGAAGATCAATGCACGGCACG

pBG028: KPI(-4→57; M15L, S17Y) — See Figure 14

1493: GTCCGTGCCGTGCTTTGATCTACCGCTGGTACTTTGACGT

1494: CAAAGTACCAGCGGTAGATCAAAGCACGGCACG

pTW6183: KPI(-4→57; I16H, S17F) — See Figure 15

925: GTCCGTGCCGTGCAATGCACTTCCGCTGGTACTTTGACGT

926: CAAAGTACCAGCGGAAGTGCATTGCACGGCACG

pTW6184: KPI(-4→57; I16H, S17Y) — See Figure 16

927: GTCCGTGCCGTGCAATGCACTACCGCTGGTACTTTGACGT

928: CAAAGTACCAGCGGTAGTGCATTGCACGGCACG

pTW6185: KPI(-4→57; I16H, S17W) — See Figure 17

929: GTCCGTGCCGTGCAATGCACTGGCGCTGGTACTTTGACGT

930: CAAAGTACCAGCGCCAGTGCATTGCACGGCACG

pTW6173: KPI(-4→57; M15A, I16H) — See Figure 18

863: GTCCGTGCCGTGCAGCTCACTCCCGCTGGTACTTTGACGT

864: CAAAGTACCAGCGGGAGTGAGCTGCACGGCACG

pTW6174: KPI(-4→57; M15L, I16H) — See Figure 19

865: GTCCGTGCCGTGCATTGCACTCCCGCTGGTACTTTGACGT

866: CAAAGTACCAGCGGGAGTGCAATGCACGGCACG

C. Transformation of yeast with expression vectors

Yeast strain ABL115 was transformed by electroporation exactly according to the protocol described for transformation by pTW113.

D. Induction of transformed yeast strains, purification of KPI(-4→57) variants.

Cultures of yeast strains were grown and induced, and recombinant secreted KPI(-4→57) variants were purified according to the procedure described for KPI(-4→57). The amino acid sequences of KPI(-4→57) variants are shown in Figures 20-29.

Example 3. Identification of KPI (-4→57; M15A, S17F) DD185 by phage display.

A. Construction of vector pSP26:Amp:F1

The construction of pSP26:*Amp*:F1 is outlined in Figure 30. Vector pSP26:*Amp*:F1 contributes the basic plasmid backbone for the construction of the phage display vector for the *phoA*:KPI fusion, PDW1 #14. pSP26:*Amp*:F1 contains a low-copy number origin of

replication, the ampicillin-resistance gene (*Amp*) and the F1 origin for production of single-stranded phagemid DNA.

The ampicillin-resistance gene (*Amp*) was generated through polymerase chain reaction (PCR) amplification from the plasmid genome of PUC19 using oligonucleotides 176 and 177.

176: GCCATCGATGGTTTCTTAAGCGTCAGGTGGCACTTTTC
177: GCGCCAATTCTTGGTCTACGGGGTCTGACGCTCAGTGGAACGAA

The PCR amplification of *Amp* was done according to standard techniques, using Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). Amplification from plasmid pUC19 with these oligonucleotides yielded a fragment of 1159 bp, containing *Pfl*MI and *Cla*I restriction sites. The PCR product was digested with *Pfl*MI and *Cla*I and purified by agarose gel electrophoresis in 3% NuSieve Agarose (FMC Corp.). Bacterial expression vector pSP26 (*supra*) was digested with *Pfl*MI and *Cla*I and the larger vector fragment was purified. The *Pfl*MI-*Cla*I PCR fragment was ligated into the previously digested pSP26 containing the *Amp* gene. The ligation product was used to transform *E. coli* strain MC1061 and colonies were selected by ampicillin resistance. The resulting plasmid is denoted pSP26:*Amp*.

The F1 origin of replication from the mammalian expression vector pcDNAII (Invitrogen) was isolated in a 692 bp *Ear*I fragment. Plasmid pcDNAII was digested with *Ear*I and the resulting 692 bp fragment purified by agarose gel electrophoresis. *Ear*I-*Not*I adapters were added to the 692 bp *Ear*I fragment by ligation of two annealed oligonucleotide pairs, 179 + 180 and 181 + 182. The oligo pairs were annealed as described above.

179: GGCCGCTCTTCC
180: AAAGGAAGAGC
181: CTAGAATTGC
182: GGCCGCAATTC

The oligonucleotide-ligated fragment was then ligated into the single *Not*I site of PSP26:*Amp* to yield the vector pSP26:*Amp*:F1.

B. Construction of vector pgIII

The construction of pgIII is outlined in Figure 31. The portion of the phage geneIII protein gene contained by the PDW1 #14 phagemid vector was originally obtained as a PCR amplification product from vector m13mp8. A portion of m13mp8 geneIII encoding the carboxyl-terminal 158 amino acid residues of the geneIII product was isolated by PCR amplification of m13mp8 nucleotide residues 2307-2781 using PCR oligos 6162 and 6160.

6162: GCCGGATCCGCTATTTCCGGTGGTGGCTCTGGTTCC
6160: GCCAAGCTTATTAAGACTCCTTATTACGCAG

The PCR oligos contain *Bam*HI and *Hin*dIII restriction recognition sites such that PCR from m13mp8 plasmid DNA with the oligo pair yielded a 490 bp *Bam*HI-*Hin*dIII fragment encoding the appropriate portion of geneIII. The PCR product was ligated between the *Bam*HI and *Hin*dIII sites within the polylinker of PUC19 to yield plasmid pgIII.

C. Construction of pPhoA:KPI:gIII

Construction of p*Pho*A:KPI:gIII is outlined in Figure 32. A portion of the *pho*A signal sequence and KPI fusion encoded by the phage display vector PDW1 #14 originates with p*Pho*A:KPI:gIII. The 237 bp *Nde*I-*Hin*dIII fragment of pTW10:KPI encoding the entire *pho*A:KPI (1→57) fusion was isolated by preparative agarose gel electrophoresis, and inserted between the *Nde*I and *Hin*dIII sites of pUC19 to yield plasmid p*Pho*A:KPI. The 490 bp *Bam*HI-*Hin*dIII fragment of pgIII encoding the C-terminal portion of the geneIII product was then isolated and ligated between the *Bam*HI and *Hin*dIII sites of p*Pho*A:KPI to yield vector p*Pho*a:KPI:gIII. The p*Pho*A:KPI:gIII vector encodes a 236 amino acid residue

fusion of the *pho*A signal peptide, KPI (1→57) and the carboxyl-terminal portion of the geneIII product.

D. Construction of pLG1

Construction of pLG1 is illustrated in Figure 33. The exact geneIII sequences contained in vector PDW1 #14 originate with phage display vector pLG1. A modified geneIII segment was generated by PCR amplification of the geneIII region from pgIII using PCR oligonucleotides 6308 and 6305.

6308: AGCTCCGATCTAGGATCCGGTGGTGGCTCTGGTTCCGGT

6305: GCAGCGGCCGTTAAGCTTATTAAGACTCCT

PCR amplification from pgIII with these oligonucleotides yielded a 481 bp *Bam*HI-*Hin*dIII fragment encoding a geneIII product shortened by 3 amino acid residues at the amino-terminal portion of the segment of the geneIII fragment encoded by pgIII. A 161 bp *Nde*I-*Bam*HI fragment was generated by PCR amplification from bacterial expression plasmid pTHW05 using oligonucleotides 6306 and 6307.

6306: GATCCTTGTGTCCATATGAAACAAAGC

6307: CACGTCGGTCGAGGATCCCTAACCACGGCCTTTAACCAG

The 161 bp *Nde*I-*Bam*HI fragment and the 481 bp *Bam*HI-*Hin*dIII fragment were gel-purified, and then ligated in a three-way ligation into PTW10 which had previously been digested with *Nde*I and *Hin*dIII. The resulting plasmid pLG1 encodes a *pho*A signal peptide-insert-geneIII fusion for phage display purposes.

E. Construction of pAL51

Construction of pAL51 is illustrated in Figure 34. Vector pAL51 contains the geneIII sequences of pLG1 which are to be incorporated in vector pDW1 #14.

A 1693 bp fragment of plasmid pBR322 was isolated, extending from the *Bam*HI site at nucleotide 375 to the *Pvu*II site at position 2064. Plasmid pLG1 was digested with *Asp*718I and *Bam*HI, removing an 87 bp fragment. The overhanging *Asp*718I end was blunted by treatment with Klenow fragment, and the *Pvu*II-*Bam*HI fragment isolated from pBR322 was ligated into this vector, resulting in the insertion of a 1693 bp "stuffer" region between the *Asp*718I and *Bam*HI sites. The 78 bp *Nde*I-*Asp*718I region of the resulting plasmid was removed and replaced with the annealed oligo pair 6512 + 6513.

6512: TATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTT
TACCCCGGTGACCAAAGCCCACGCTGAAG

6513: GTACCTTCAGCGTGGGCTTTGGTCACCGGGGTAAACAGTAACGGT
AAGAGTGCCAGTGCAATAGTGCTTTGTTTCA

The newly created 74 bp *Nde*I-*Asp*718I fragment encodes the *pho*A signal peptide, and contains a *Bst*EII cloning site. The resulting plasmid is denoted pAL51.

F. Construction of pAL53

Construction of pAL53 is outlined in Figure 35. Plasmid pAL53 contributes most of the vector sequence of pDW1 #14, including the basic vector backbone with *Amp* gene, F1 origin, low copy number origin of replication, geneIII segment, *pho*A promotor and *pho*A signal sequence.

Plasmid pAL51 was digested with *Nde*I and *Hin*dIII and the resulting 2248 bp *Nde*I-*Hin*dIII fragment encoding the *pho*A signal peptide, stuffer region and geneIII region was isolated by preparative agarose gel electrophoresis. The *Nde*I-*Hin*dIII fragment was ligated into plasmid pSP26:*Amp*:F1 between the *Nde*I and *Hin*dIII sites, resulting in plasmid pAL52.

The *pho*A promoter region and signal peptide was generated by amplification of a portion of the *E. coli* genome by PCR, using oligonucleotide primers 405 and 406.

405: CCGGACGCGTGGAGATTATCGTCACTG

406: GCTTTGGTCACCGGGGTAAACAGTAACGG

The resulting PCR product is a 332 bp *Mlu*I-*Bst*EII fragment which contains the *pho*A promoter region and signal peptide sequence. This fragment was used to replace the 148 bp *Mlu*I-*Bst*EII segment of PAL52, resulting in vector pAL53.

G. Construction of pSP26:Amp:F1:PhoA:KPI:gIII

Construction of pSP26:*Amp*:F1:*Pho*A:KPI:gIII is illustrated in Figure 36. This particular vector is the source of the KPI coding sequence found in vector pDW1 #14. Plasmid p*Phoa*:KPI:gIII was digested with *Nde*I and *Hin*dIII, and the resulting 714 bp *Nde*I-*Hin*dIII fragment was purified, and then inserted into vector pSP26:*Amp*:F1 between the *Nde*I and *Hin*dIII sites. The resulting plasmid is denoted pSP26:*Amp*:F1:*Pho*A:KPI:gIII.

H. Construction of pDW1 #14

Construction of pDW1 #14 is illustrated in Figure 37. The sequences encoding KPI were amplified from plasmid pSP26:*Amp*:F1:*Pho*A:KPI:gIII by PCR, using oligonucleotide primers 424 and 425.

424: CTGTTTACCCCGGTGACCAAAGCCGAGGTGTGCTCTGAACAA

425: AATAGCGGATCCGCACACTGCCATGCAGTACTCTTC

The resulting 172 bp *Bst*EII-*Bam*HI fragment encodes most of KPI (1→55). This fragment was used to replace the stuffer region in pAL53 between the *Bst*EII and *Bam*HI sites. The resulting plasmid, PDW1 #14, is the parent KPI phage display vector for preparation of randomized KPI phage libraries. The coding region for the *pho*A-KPI (1→55)-geneIII fusion is shown in Figure 38.

I. Construction of pDW1 14-2

Construction of pDW1 14-2 is illustrated in Figure 39. The first step in the construction of the KPI phage libraries in pDW1 #14 was the replacement of the *Age*I-*Bam*HI fragment within the KPI coding sequence with a stuffer fragment. This greatly aids in preparation of randomized KPI libraries which are substantially free of contamination of phagemid genomes encoding wild-type KPI sequence.

Plasmid pDW1 #14 was digested with *Age*I and *Bam*HI, and the 135 bp *Age*I-*Bam*HI fragment encoding KPI was discarded. A stuffer fragment was created by PCR amplification of a portion of the PBR322 *Tet* gene, extending from the *Bam*HI site at nucleotide 375 to nucleotide 1284, using oligo primers 266 and 252.

266: GCTTTAAACCGGTAGGTGGCCCGGCTCCATGCACC
252: CGAATTCACCGGTGTCATCCTCGGCACCGTCACCCT

The resulting 894 bp *Age*I-*Bam*HI stuffer fragment was then inserted into the *Age*I/*Bam*HI-digested pDW1 #14 to yield the phagemid vector pDW1 14-2. This vector was the starting point for construction of the randomized KPI libraries.

J. Construction of KPI Library 16-19

Construction of KPI Library 16-19 is outlined in Figure 40. Library 16-19 was constructed to display KPI-geneIII fusions in which amino acid positions Ala^{14}, Met^{15}, Ile^{16} and Ser^{17} are randomized. For preparation of the library, plasmid pDW1 14-2 was digested with *Age*I and *Bam*HI to remove the stuffer region, and the resulting vector was purified by preparative agarose gel electrophoresis. Plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the *Age*I site to the *Bam*HI site. The oligonucleotide primers used were 544 and 551.

544: GGGCTGAGACCGGTCCGTGCCGT$(NNS)_4$CGCTGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 544 contains four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop codon, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp *Age*I-*Bam*HI fragments all containing different sequences in the randomized region. The PCR product was purified by preparative agarose gel electrophoresis and ligated into the *Age*I/*Bam*HI digested PDW1 14-2 vector. The ligation mixture was used to transform *E. coli* Top10F' cells (Invitrogen) by electroporation according to the manufacturer's directions. The resulting Library 16-19 contained approximately 400,000 independent clones. The potential size of the library, based upon the degeneracy of the priming PCR oligo #544 was 1,048,576 members. The expression unit encoded by the members of Library 16-19 is shown in Figure 41.

K. Selection of Library 16-19 with human plasma kallikrein

KPI phage were prepared and amplified by infecting transformed cells with M13KO7 helper phage as described by Matthews *et al.*, *Science* 260:1113 (1993). Human plasma kallikrein (Enzyme Research Laboratories, South Bend, IN), was coupled to Sepharose 6B resin. Prior to phage binding, the immobilized kallikrein resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, $CaCl_2$, $MgCl_2$, 0.1% gelatin, and 0.05% Triton X-100). Approximately $5x10^9$ phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were bound to 50 μl kallikrein resin containing 15 pmoles of active human plasma kallikrein in a total volume of 250 μl. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the kallikrein resin three times

in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After three rounds of selection on kallikrein-Sepharose, phagemid DNA was isolated from 22 individual colonies and subjected to DNA sequence analysis.

The most frequently occurring randomized KPI region encoded: Ala^{14}-Ala^{15}-Ile^{16}-Phe^{17}. The *pho*A-KPI-geneIII region encoded by this class of selected KPI phage is shown in Figure 42. The KPI variant encoded by these phagemids is denoted KPI (1→55; M15A, S17F).

L. Construction of pDD185 KPI (-4→57; M15A, S17F)

Figure 43 outlines the construction of pDD185 KPI (-4→57; M15A, S17F). The sequences encoding KPI (1→55; M15A, S17F) were moved from one phagemid vector, pDW1 (16-19) 185, to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Age*I and *Bam*HI and the 135 bp *Age*I-*Bam*HI fragment was discarded. The 135 bp *Age*I-*Bam*HI fragment of pDW1 (16-19) 185 was isolated and ligated into the yeast vector to yield plasmid pDD185, encoding α-factor fused to KPI (-4→57; M15A, S17F). See Figure 44.

M. Purification of KPI (-4→57; M15A, S17F) pDD185

Transformation of yeast strain ABL115 with pDD185, induction of yeast cultures, and purification of KPI (-4→57; M15A, S17F) pDD185 was accomplished as described for the other KPI variants.

N. Construction of KPI Library 6 — M15A, with residues 14, 16-18 random.

Library 6 was constructed to display KPI-geneIII fusions in which amino acid positions Ala^{14}, Ile^{16}, Ser^{17}

and Arg^{18} are randomized, but position 15 was held constant as Ala. For preparation of the library, plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the *Age*I site to the *Bam*HI site. The oligonucleotide primers used were 551 and 1003.

1003: GCTGAGACCGGTCCGTGCCGTNNSGCA(NNS)$_3$TGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1003 contained four randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encodes all 20 amino acids plus a single possible stop, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp *Age*I-*Bam*HI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with *Bam*HI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with *Bam*HI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector which was then digested with *Age*I to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately 5x10^6 independent clones.

O. Construction of KPI Library 7 — residues 14-18 random.

Library 7 was constructed to display KPI-geneIII fusions in which amino acid positions Ala^{14}, Met^{15}, Ile^{16}, Ser^{17} and Arg^{18} are randomized. For preparation of the library, plasmid pDW1 #14 was used as template in a PCR amplification of the KPI region extending from the *Age*I site to the *Bam*HI site. The oligonucleotide primers used were 551 and 1179.

1179: GCTGAGACCGGTCCGTGCCGT(NNS)$_5$TGGTACTTTGACGTC

551: GGAATAGCGGATCCGCACACTGCCATGCAG

Oligonucleotide primer 1179 contains five randomized codons of the sequence NNS, where N represents equal mixtures of A/G/C/T and S an equal mixture of G or C. Each NNS codon thus encoded all 20 amino acids plus a single possible stop, in 32 different DNA sequences. PCR amplification from the wild-type KPI gene resulted in the production of a mixture of 135 bp *Age*I-*Bam*HI fragments all containing different sequences in the randomized region. The PCR product was phenol extracted, ethanol precipitated, digested with *Bam*HI and purified by preparative agarose gel electrophoresis. Plasmid pDW1 14-2 was digested with *Bam*HI, phenol extracted and ethanol precipitated. The insert was ligated at high molar ratio to the vector which was then digested with *Age*I to remove the stuffer region. The vector containing the insert was purified by agarose gel electrophoresis and recircularized. The resulting library contains approximately $1x10^7$ independent clones.

P. *Selection of Libraries 6 & 7 with human factor XIIa*

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor XIIa (Enzyme Research Laboratories, South Bend, IN), was biotinylated as follows. Factor XIIa (0.5 mg) in 5mM sodium acetate pH 8.3 was incubated with Biotin Ester (Zymed) at room temperature for 1.5 h, then buffer-exchanged into assay buffer (AB). Approximately $1x10^{10}$ phage particles of each amplified Library 6 or 7 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were incubated with 50 pmoles of active biotinylated human factor XIIa in a total volume of 200 µl. Phage were allowed to bind for 2 h at room temperature, with rocking. Following the binding period, 100 µl Strepavidin Magnetic Particles (Boehringer

Mannheim) were added to the mixture and incubated at room temperature for 30 minutes. Separation of magnetic particles from the supernatant and wash/elution buffers was carried out using MPC-E-1 Neodymium-iron-boron permanent magnets (Dynal). Unbound phage were removed by washing the magnetically bound biotinylated XIIa-phage complexes three times with 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0, 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After 3 or 4 rounds of selection with factor XIIa, phagemid DNA was isolated from individual colonies and subjected to DNA sequence analysis.

Sequences in the randomized regions were compared with one another to identify consensus sequences appearing more than once. From Library 6 a phagemid was identified which encoded M15L, S17Y, R18H. From Library 7 a phagemid was identified which encoded M15A, S17Y, R18H.

Q. *Construction of pBG015 KPI (-4→57; M15L, S17Y, R18H), pBG022 (-4→57; M15A, S17Y, R18H)*

The sequences encoding KPI (1→55; M15L, S17Y, R18H) and KPI (1→55; M17A, S17Y, R18H) were moved from the phagemid vectors to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Age*I and *Bam*HI and the 135 bp *Age*I-*Bam*HI fragment was discarded. The 135 bp *Age*I-*Bam*HI fragment of the phagemid vectors were isolated and ligated into the yeast vector to yield plasmids pBG015 and pBG022, encoding alpha-factor fused to KPI (-4→57; M15L, S17Y, R18H), and KPI (-4→57; M15A, S17Y, R18H), respectively.

R. *Construction of pBG029 KPI (-4→57, T9V, M15L, S17Y, R18H)*

Plasmid pBG015 was digested with *Xba*I and *Rsr*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously.

1593: CTAGATAAAAGAGAGGTTGTTAGAGAGGTGTGCTCTGAACAAGCT GAGGTTG

1642: GACCAACCTCAGCTTGTTCAGAGCACACCTCTCTAA CAACCTCTCTTTTAT

The annealed oligonucleotides were ligated into the *Xba*I and *Rsr*II-digested pBG015, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG029, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; T9V, M15L, S17F, R18H) fusion.

S. *Construction of pBG033 KPI (-4→57; T9V, M15A, S17Y, R18H)*

Plasmid pBG022 was digested with *Xba*I and *Rsr*II, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (1593 + 1642) was phosphorylated, annealed and gel-purified as described previously. The annealed oligonucleotides were ligated into the *Xba*I and *Rsr*II-digested pBG022, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pBG033, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; T9V, M15A, S17F, R18H) fusion.

T. *Selection of Library 16-19 with human factor Xa*

KPI phage were prepared and amplified by infecting transformed cells with M13K07 helper phage (Matthews and Wells, 1993). Human factor Xa (Haematologic Technologies, Inc., Essex Junction, VT) was coupled to Sepharose 6B resin. Prior to phage binding, the

immobilized Xa resin was washed three times with 0.5 ml assay buffer (AB = 100mM Tris-HCl, pH 7.5, 0.5M NaCl, 5mM each of KCl, $CaCl_2$, $MgCl_2$, 0.1% gelatin, and 0.05% Triton X-100). Approximately 4×10^{10} phage particles of the amplified Library 16-19 in PBS, pH 7.5, containing 300mM NaCl and 0.1% gelatin, were bound to 50 µl Xa resin in a total volume of 250 µl. Phage were allowed to bind for 4 h at room temperature, with rocking. Unbound phage were removed by washing the Xa resin three times in 0.5 ml AB. Bound phage were eluted sequentially by successive 5 minute washes: 0.5 ml 50mM sodium citrate, pH 6.0, 150mM NaCl; 0.5 ml 50mM sodium citrate, pH 4.0 150mM NaCl; and 0.5 ml 50mM glycine, pH 2.0, 150mM NaCl. Eluted phage were neutralized immediately and phagemids from the pH 2.0 elution were titered and amplified for reselection. After three rounds of selection on Xa-Sepharose, phagemid DNA was isolated and subjected to DNA sequence analysis.

Sequences in the randomized Ala^{14}-Ser^{17} region were compared with one another to identify consensus sequences appearing more than once. A phagemid was identified which encoded KPI (1→55; M15L, I16F, S17K).

U. Construction of pDD131 KPI (-4→57; M15L, I16F, S17K)

The sequences encoding KPI (1→55; M15L, I16F, S17K) were moved from the phagemid vector to the yeast expression vector so that the KPI variant could be purified and tested.

Plasmid pTW113 encoding wild-type KPI (-4→57) was digested with *Age*I and *Bam*HI and the 135 bp *Age*I-*Bam*HI fragment was discarded. The 135 bp *Age*I-*Bam*HI fragment of the phagemid vector was isolated and ligated into the yeast vector to yield plasmid pDD131, encoding alpha-factor fused to KPI (-4→57; M15L, I16F, S17K).

V. Construction of pDD134 KPI (-4→57; M15L, I16F, S17K, G37Y)

Plasmid pDD131 was digested with *Aat*I and *Bam*HI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (738 + 739) was phosphorylated, annealed and gel-purified as described previously.

738: CACTGAAGGTAAGTGCGCTCCATTCTTTTACGGCGGTTGCTACGGCAACCGT
AACAACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

739: GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACGGTTGC
CGTAGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGACGT

The annealed oligonucleotides were ligated into the *Aat*I and *Bam*HI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD134, encodes the 445 bp synthetic gene for the alpha-factor-KPI (-4→57; M15L, I16F, S17K, G37Y) fusion.

W. Construction of pDD135 KPI (-4→57; M15L, I16F, S17K, G37L)

Plasmid pDD131 was digested with *Aat*II and *Bam*HI, and the larger of the two resulting fragments was isolated. An oligonucleotide pair (724 + 725) was phosphorylated, annealed and gel-purified as described previously.

738: CACTGAAGGTAAGTGCGCTCCATTCTTTTACGGCGGTTGCTACGGCAACCGT
AACAACTTTGACACTGAAGAGTACTGCATGGCAGTGTGCG

739: GATCCGCACACTGCCATGCAGTACTCTTCAGTGTCAAAGTTGTTACGGTTGC
CGTAGCAACCGCCGTAAAAGAATGGAGCGCACTTACCTTCAGTGACGT

The annealed oligonucleotides were ligated into the *Aat*II and *Bam*HI-digested pDD131, and the ligation product was used to transform *E. coli* strain MC1061 to ampicillin resistance. The resulting plasmid pDD135, encodes the

445 bp synthetic gene for the alpha-factor-KPI (-4→57; M15L, I16F, S17K, G37L) fusion.

Example 4. Kinetic analysis of KPI(-4→57) variants

The concentrations of active human plasma kallikrein, factor XIIa, and trypsin were determined by titration with p-nitrophenyl p'-guanidinobenzoate as described by Bender *et al., supra*, and Chase *et al., Biochem. Biophys. Res. Commun.* 29:508 (1967). Accurate concentrations of active KPI(-4→57) inhibitors were determined by titration of the activity of a known amount of active-site-titrated trypsin. For testing against kallikrein and trypsin, each KPI(-4→57) variant (0.5 to 100nM) was incubated with protease in low-binding 96-well microtiter plates at 30°C for 15-25 min, in 100mM Tris-HCl, pH 7.5, with 500mM NaCl, 5mM KCl, 5mM CaCl2, 5mM MgCl2, 0.1% Difco gelatin, and 0.05% Triton X-100. Chromogenic synthetic substrate was then be added, and initial rates at 30°C recorded by the SOFTmax kinetics program via a THERMOmax microplate reader (Molecular Devices Corp., Menlo Park, CA). The substrates used were N-α-benzoyl-L-Arg p-nitroanilide (1mM) for trypsin (20nM), and N-benzoyl-Pro-Phe-Arg p-nitroanilide (0.3mM) for plasma kallikrein (1nM). The Enzfitter (Elsevier) program was used both to plot fractional activity (i.e., activity with inhibitor, divided by activity without inhibitor), a, versus total concentration of inhibitor, I_t, and to calculate the dissociation constant of the inhibitor (K_i) by fitting the curve to the following equation:

$$a=1-\frac{[E]_t+[I]_t+K_i-\sqrt{([E]_t+[I]_t+K_i)^2-4[E]_t[I]_t}}{2[E]_t}$$

The K_is determined for purified KPI variants are shown in Figure 45. The most potent variants, KPI (-4→57; M15A, S17F) DD185 and KPI (-4→57; M15A, S17Y) TW6166 are 115-fold and 100-fold more potent,

respectively, as a human kallikrein inhibitor than wild-type KPI (-4→57). The least potent variant, KPI (-4→57; I16H, S17W) TW6185 is still 35-fold more potent than wild-type KPI.

For testing against factor XIIa, essentially the same reaction conditions were used, except that the substrate was N-benzoyl-Ile-Glu-Gly-Arg p-nitroaniline hydrochloride and its methyl ester (obtained from Pharmacia Hepar, Franklin, OH), and corn trypsin inhibitor (Enzyme Research Laboratories, South Bend, IN) was used as a control inhibitor. Factor XIIa was also obtained from Enzyme Research Laboratories.

Various data for inhibition of the serine proteases of interest kallikrein, plasmin, and factors Xa, XIa, and XIIa by a series of KPI variants are given in Figure 46. The results indicate that KPI variants can be produced that can bind to and preferably inhibit the activity of serine proteases. The results also indicate that the peptides of the invention may exhibit the preferable more potent and specific inhibition of one or more serine proteases of interest.

Example 5. Effect of KPI variant KPI185-1 on postoperative bleeding

A randomized, double-blinded study using an acute porcine cardiopulmonary bypass (CPB) model was used to investigate the effect of KPI185-1 on postoperative bleeding. Sixteen pigs (55-65 kg) underwent 60 minutes of hypothermic (28°C) open-chest CPB with 30 minutes of cardioplegic cardiac arrest. Pigs were randomized against a control solution of physiological saline (NS; n=8) or KPI-185 (n=8) groups. During aortic cross-clamping, the tricuspid valve was inspected through an atriotomy which was subsequently repaired. Following reversal of heparin with protamine, dilateral thoracostomy tubes were placed and shed blood collected for 3 hours. Shed blood volume and hemoglobin (Hgb) loss were calculated from total chest tube output and residual intrathoracic blood at time of sacrifice.

Total blood loss was significantly reduced in the KPI185-1 group (245.75 ± 66.24 ml vs. 344.25 ± 63.97 ml, $p=0.009$). In addition, there was a marked reduction in total Hgb loss in the treatment group (13.59 ± 4.26 gm vs. 23.61 ± 4.69 gm, $p=0.0005$). Thoracostomy drainage Hgb was significantly increased at 30 and 60 minutes in the control group [6.89 ± 1.44 vs. 4.41 ± 1.45 gm/dl ($p=0.004$) and 7.6 ± 1.03 vs. 5.26 ± 1.04 gm/dl ($p=0.0002$), respectively]. Preoperative and post-CPB hematocrits were not statistically different between the groups. These results are shown in graphical form in Figures 47-50.

The invention has been disclosed broadly and illustrated in reference to representative embodiments described above. Those skilled in the art will recognize that various modifications can be made to the present invention without departing from the spirit and scope thereof.

What Is Claimed Is:

1. A protease inhibitor comprising the sequence:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-X^3-Cys-Arg-Ala-X^4-X^5-X^6-X^7-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-X^8-Tyr-Gly-Gly-Cys-X^9-X^{10}-X^{11}-X^{12}-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu;

X^2 is selected from Thr, Val, Ile and Ser;

X^3 is selected from Pro and Ala;

X^4 is selected from Arg, Ala, Leu, Gly, or Met;

X^5 is selected from Ile, His, Leu, Lys, Ala, or Phe;

X^6 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X^7 is selected from Arg, His, or Ala;

X^8 is selected from Phe, Val, Leu, or Gly;

X^9 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr;

X^{10} is selected from Ala, Arg, or Gly;

X^{11} is selected from Lys, Ala, or Asn;

X^{12} is selected from Ser, Ala, or Arg;

provided that:

when X^4 is Arg, X^6 is Ile;

when X^9 is Arg, X^4 is Ala or Leu; when X^9 is Tyr, X^4 is Ala or X^5 is His; and

either X^5 is not Ile; or X^6 is not Ser; or X^9 is not Leu, Phe, Met, Tyr, or Asn; or X^{10} is not Gly; or X^{11} is not Asn; or X^{12} is not Arg.

2. A protease inhibitor comprising the sequence:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X^2-X^3-X^4-Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X^5-Gly-Asn-Arg-Asn-

Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-
Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu;

X^2 is selected from Ala, Leu, Gly, or Met;

X^3 is selected from Ile, His, Leu, Lys, Ala, or Phe;

X^4 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X^5 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr;

provided that:

when X^5 is Arg, X^2 is Ala or Leu; when X^5 is Tyr, X^2 is Ala or X^3 is His; and

either X^3 is not Ile; or X^4 is not Ser; or X^5 is not Leu, Phe, Met, Tyr, or Asn.

3. A protease inhibitor comprising the sequence:

Glu-Val-Val-Arg-Glu-Val-Cys-Ser-Glu-Gln-
Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-X^1-X^2-X^3-
Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-
Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X^4-
Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-
Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X^1 is selected from Ala, Leu, Gly, or Met;

X^2 is selected from Ile, His, Leu, Lys, Ala, or Phe;

X^3 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X^4 is selected from Gly, Arg, Leu, Met, or Tyr;

provided that:

when X^1 is Ala, X^2 is Ile, His, or Leu;

when X^1 is Leu, X^2 is Ile or His;

when X^1 is Leu and X^2 is Ile, X^3 is not Ser;

when X^1 is Gly, X^2 is Ile;

when X^4 is Arg, X^1 is Ala or Leu;

when X^4 is Tyr, X^1 is Ala or X^2 is His; and

either X^1 is not Met, or X^2 is not Ile, or X^3 is not Ser, or X^4 is not Gly.

4. A protease inhibitor according to claim 1, wherein at least two amino acid residues selected from the group consisting of X^4, X^5, X^6, and X^7 differ from the residues found in the naturally occurring sequence of KPI.

5. A protease inhibitor according to claim 1, wherein X^1 is Asp or Glu, X^2 is Thr, X^3 is Pro, and X^{12} is Ser.

6. A protease inhibitor according to claim 5, wherein X^1 is Glu, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Gly, and X^{11} is Asn.

7. A protease inhibitor according to claim 5, wherein X^1 is Asp, X^2 is Thr, X^3 is Pro, X^4 is Arg, X^5 is Ile, X^6 is Ile, X^7 is Arg, x^8 is Val, X^9 is Arg, X^{10} is Ala, and X^{11} is Lys.

8. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Ala.

9. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg.

10. A protease inhibitor according to claim 1, wherein X^1 is Glu, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Ala, X^{11} is Asn, and X^{12} is Arg.

11. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Phe, X^9 is Gly, X^{10} is Arg, X^{11} is Asn, and X^{12} is Arg.

12. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Arg, x^8 is Val, Leu, or Gly, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg.

13. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Met, X^5 is Ile, X^6 is Ser, X^7 is Ala, x^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Asn, and X^{12} is Arg.

14. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Pro, X^4 is Ala or Leu, X^5 is Ile, X^6 is Tyr, X^7 His, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg.

15. A protease inhibitor according to claim 14, wherein X^2 is Thr, and X^4 is Ala.

16. A protease inhibitor according to claim 14, wherein X^2 is Thr, and X^4 is Leu.

17. A protease inhibitor according to claim 14, wherein X^2 is Val, and X^4 is Ala.

18. A protease inhibitor according to claim 14, wherein X^2 is Ser, and X^4 is Ala.

19. A protease inhibitor according to claim 14, wherein X^2 is Val, and X^4 is Leu.

20. A protease inhibitor according to claim 14, wherein X^2 is Ser, and X^4 is Leu.

21. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Leu, X^5 is Phe, X^6 is Lys, X^7 is Arg, X^8 is Phe, X^9 is Gly, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg.

22. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Leu, X^5 is Phe, X^6 is Lys, X^7 is Arg, X^8 is Phe, X^9 is Tyr, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg.

23. A protease inhibitor according to claim 1, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Pro, X^4 is Leu, X^5 is Phe, X^6 is Lys, X^7 is Arg, X^8 is Phe, X^9 is Leu, X^{10} is Gly, X^{11} is Ala, and X^{12} is Arg.

24. A protease inhibitor according to claim 2, wherein X^1 is Glu, X^2 is Met, X^3 is Ile, X^4 is Ile, and X^5 is Gly.

25. A protease inhibitor according to claim 3, wherein X^1 is Met, X^3 is Ser, and X^4 is Gly.

26. A protease inhibitor according to claim 25, wherein X^2 is selected from His, Ala, Phe, Lys, and Leu.

27. A protease inhibitor according to claim 26, wherein X^2 is His.

28. A protease inhibitor according to claim 27, wherein X^2 is Ala.

29. A protease inhibitor according to claim 27, wherein X^2 is Phe.

30. A protease inhibitor according to claim 27, wherein X^2 is Lys.

31. A protease inhibitor according to claim 27, wherein X^2 is Leu.

32. A protease inhibitor according to claim 3, wherein X^1 is Met, X^2 is Ile, and X^4 is Gly.

33. A protease inhibitor according to claim 32, wherein X^3 is Ile.

34. A protease inhibitor according to claim 32, wherein X^3 is Pro.

35. A protease inhibitor according to claim 32, wherein X^3 is Phe.

36. A protease inhibitor according to claim 32, wherein X^3 is Tyr.

37. A protease inhibitor according to claim 32, wherein X^3 is Trp.

38. A protease inhibitor according to claim 32, wherein X^3 is Asn.

39. A protease inhibitor according to claim 32, wherein X^3 is Leu.

40. A protease inhibitor according to claim 32, wherein X^3 is Lys.

41. A protease inhibitor according to claim 32, wherein X^3 is His.

42. A protease inhibitor according to claim 32, wherein X^3 is Glu.

43. A protease inhibitor according to claim 3, wherein X^1 is Ala.

44. A protease inhibitor according to claim 43, wherein X^2 is Ile.

45. A protease inhibitor according to claim 44, wherein X^3 is Phe, and X^4 is Gly.

46. A protease inhibitor according to claim 44, wherein X^3 is Tyr, and X^4 is Gly.

47. A protease inhibitor according to claim 44, wherein X^3 is Trp, and X^4 is Gly.

48. A protease inhibitor according to claim 44, wherein X^3 is Ser or Phe, and X^4 is Arg or Tyr.

49. A protease inhibitor according to claim 43, wherein X^2 is His or Leu, X^3 is Phe, and X^4 is Gly.

50. A protease inhibitor according to claim 3, wherein X^1 is Leu.

51. A protease inhibitor according to claim 50, wherein X^2 is His, X^3 is Asn or Phe, and X^4 is Gly.

52. A protease inhibitor according to claim 50, wherein X^2 is Ile, X^3 is Pro, and X^4 is Gly.

53. A protease inhibitor according to claim 3, wherein X^1 is Gly, X^2 is Ile, X^3 is Tyr, and X^4 is Gly.

54. A protease inhibitor according to claim 3, wherein X^1 is Met, X^2 is His, X^3 is Ser, and X^4 is Tyr.

55. An isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 1.

56. An isolated DNA molecule according to claim 55, operably linked to a regulatory sequence that controls expression of the coding sequence in a host cell.

57. An isolated DNA molecule according to claim 56, further comprising a DNA sequence encoding a secretory signal peptide.

58. An isolated DNA molecule according to claim 57, wherein said secretory signal peptide comprises the signal sequence of yeast alpha-mating factor.

59. A host cell transformed with a DNA molecule according to claim 55.

60. A host cell according to claim 59, wherein said host cell is *E. coli* or a yeast cell.

61. A host cell according to claim 60, wherein said yeast cell is selected from *Pichia pastoris* and *Saccharomyces cerevisiae*.

62. A method for producing a protease inhibitor, comprising the steps of culturing a host cell according to claim 59 and isolating and purifying said protease inhibitor.

63. A pharmaceutical composition, comprising a protease inhibitor according to claim 1, together with a pharmaceutically acceptable sterile vehicle.

64. A method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition according to claim 63.

65. The method of treatment of claim 64, wherein said clinical condition is blood loss during surgery.

66. A method for inhibiting the activity of serine proteases of interest in a mammal comprising administering a therapeutically effective dose of a pharmaceutical composition according to claim 63.

67. The method of claim 66, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

68. A protease inhibitor comprising the sequence:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-X^2-Gly-Pro-Cys-Arg-Ala-X^3-X^4-X^5-X^6-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-X^7-Gly-Asn-Arg-Asn-Asn-Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-Cys-Gly-Ser-Ala-Ile,

wherein:

X^1 is selected from Glu-Val-Val-Arg-Glu-, Asp, or Glu;

X^2 is selected from Thr, Val, Ile and Ser;

X^3 is selected from Arg, Ala, Leu, Gly, or Met;

X^4 is selected from Ile, His, Leu, Lys, Ala, or Phe;

X^5 is selected from Ser, Ile, Pro, Phe, Tyr, Trp, Asn, Leu, His, Lys, or Glu;

X^6 is selected from Arg, His, or Ala; and

X^7 is selected from Gly, Ala, Lys, Pro, Arg, Leu, Met, or Tyr.

69. A protease inhibitor according to claim 68, wherein at least two amino acid residues selected from the group consisting of X^3, X^4, X^5, and X^6 differ from the residues found in the naturally occurring sequence of KPI.

70. A protease inhibitor according to claim 68, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, Val, or Ser, X^3 is Ala or Leu, X^4 is Ile, X^5 is Tyr, X^6 is His and X^7 is Gly.

71. A protease inhibitor according to claim 70, wherein X^2 is Thr, and X^3 is Ala.

72. A protease inhibitor according to claim 70, wherein X^2 is Thr, and X^3 is Leu.

73. A protease inhibitor according to claim 70, wherein X^2 is Val, and X^3 is Ala.

74. A protease inhibitor according to claim 70, wherein X^2 is Ser, and X^3 is Ala.

75. A protease inhibitor according to claim 70, wherein X^2 is Val, and X^3 is Leu.

76. A protease inhibitor according to claim 70, wherein X^2 is Ser, and X^3 is Leu.

77. A protease inhibitor according to claim 68, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Leu, X^4 is Phe, X^5 is Lys, X^6 is Arg and X^7 is Gly.

78. A protease inhibitor according to claim 68, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Leu, X^4 is Phe, X^5 is Lys, X^6 is Arg and X^7 is Tyr.

79. A protease inhibitor according to claim 68, wherein X^1 is Glu-Val-Val-Arg-Glu-, X^2 is Thr, X^3 is Leu, X^4 is Phe, X^5 is Lys, X^6 is Arg and X^7 is Leu.

80. A protease inhibitor comprising the sequence:

X^1-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-
Pro-Cys-X^2-Ala-X^3-X^4-X^5-X^6-Trp-Tyr-Phe-Asp-
Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-
Tyr-Gly-Gly-Cys-Gly-Gly-Asn-Arg-Asn-Asn-
Phe-Asp-Thr-Glu-Glu-Tyr-Cys-Met-Ala-Val-
Cys-Gly-Ser-Ala-Ile,

wherein:

$X^{1'}$ is selected from Glu-Val-Val-Arg-Glu- and Asp-Val-Val-Arg-Glu-;

X^2 is selected from Arg and Lys;

X^3 is selected from Met, Arg, Ala, Leu, Ser, Val;

X^4 is selected from Ile and Ala;

X^5 is selected from Ser, Ile, Ala, Pro, Phe, Tyr, and Trp; and

X^6 is selected from Arg, Ala, His, Gln, and Thr;

provided that:

when X^2 is Arg, X^3 is Leu, and X^4 is Ile, X^5 cannot be Ser; and also provided that either X^3 is not Met; or X^4 is not Ile; or X^5 is not Ser; or X^6 is not Arg.

81. A protease inhibitor according to claim 80, wherein X^5 is selected from Phe, Tyr and Trp.

82. A protease inhibitor according to claim 80, wherein X^4 is Ile.

83. A protease inhibitor according to claim 82, wherein X^2 is Lys.

84. A protease inhibitor according to claim 83, wherein X^3 is Met.

85. A protease inhibitor according to claim 84, wherein X^5 is Ser.

86. A protease inhibitor according to claim 84, wherein X^5 is Ile.

87. A protease inhibitor according to claim 83, wherein X^3 is Arg.

88. A protease inhibitor according to claim 87, wherein X^5 is Ser.

89. A protease inhibitor according to claim 87, wherein X^5 is Ile.

90. A protease inhibitor according to claim 82, wherein X^2 is Arg.

91. A protease inhibitor according to claim 90, wherein X^3 is Arg or Met, and X^5 is Ser or Ile.

92. A protease inhibitor according to claim 91, wherein X^3 is Arg.

93. A protease inhibitor according to claim 92, wherein X^5 is Ser.

94. A protease inhibitor according to claim 92, wherein X^5 is Ile.

95. A protease inhibitor according to claim 91, wherein X^3 is Met.

96. A protease inhibitor according to claim 95, wherein X^5 is Ser.

97. A protease inhibitor according to claim 95, wherein X^5 is Ile.

98. A protease inhibitor according to claim 82, wherein X^3 is Ala.

99. A protease inhibitor according to claim 82, wherein X^3 is Leu.

100. A protease inhibitor according to claim 82, wherein X^3 is Ser.

101. A protease inhibitor according to claim 82, wherein X^3 is Val.

102. A protease inhibitor according to claim 82, wherein X^5 is Pro.

103. A protease inhibitor according to claim 82, wherein X^5 is Phe.

104. A protease inhibitor according to claim 82, wherein X^5 is Tyr.

105. A protease inhibitor according to claim 82, wherein X^5 is Trp.

106. A protease inhibitor according to claim 104, wherein X^3 is Ala or Leu.

107. A protease inhibitor according to claim 106, wherein X^3 is Ala.

108. A protease inhibitor according to claim 106, wherein X^3 is Leu.

109. A protease inhibitor according to claim 105, wherein X^3 is Ala.

110. A protease inhibitor according to claim 109, wherein X^5 is His.

111. A protease inhibitor according to claim 109, wherein X^5 is Gln.

112. A protease inhibitor according to claim 109, wherein X^5 is Thr.

113. An isolated DNA molecule comprising a DNA sequence encoding a protease inhibitor according to claim 80.

114. An isolated DNA molecule according to claim 113, operably linked to a regulatory sequence that controls expression of the coding sequence in a host cell.

115. An isolated DNA molecule according to claim 114, further comprising a DNA sequence encoding a secretory signal peptide.

116. An isolated DNA molecule according to claim 115, wherein said secretory signal peptide comprises the signal sequence of yeast alpha-mating factor.

117. A host cell transformed with a DNA molecule according to claim 113.

118. A host cell according to claim 117, wherein said host cell is *E. coli* or a yeast cell.

119. A host cell according to claim 118, wherein said yeast cell is selected from *Pichia pastoris* and *Saccharomyces cerevisiae*.

120. A method for producing a protease inhibitor, comprising the steps of culturing a host cell according to claim 117 and isolating and purifying said protease inhibitor.

121. A pharmaceutical composition, comprising a protease inhibitor according to claim 80, together with a pharmaceutically acceptable sterile vehicle.

122. A method of treatment of a clinical condition associated with increased activity of one or more serine proteases, comprising administering to a patient suffering from said clinical condition an effective amount of a pharmaceutical composition according to claim 121.

123. The method of treatment of claim 122, wherein said clinical condition is blood loss during surgery.

124. A method for inhibiting the activity of serine proteases of interest in a mammal comprising

administering a therapeutically effective dose of a pharmaceutical composition according to claim 121.

125. The method of claim 124, wherein said serine proteases are selected from the group consisting of: kallikrein; chymotrypsins A and B; trypsin; elastase; subtilisin; coagulants and procoagulants, particularly those in active form, including coagulation factors such as factors VIIa, IXa, Xa, XIa, and XIIa; plasmin; thrombin; proteinase-3; enterokinase; acrosin; cathepsin; urokinase; and tissue plasminogen activator.

126. A protease inhibitor according to claim 81, wherein X^4 is Ile.

127. A protease inhibitor according to claim 126, wherein X^5 is Phe.

128. A protease inhibitor according to claim 126, wherein X^5 is Tyr.

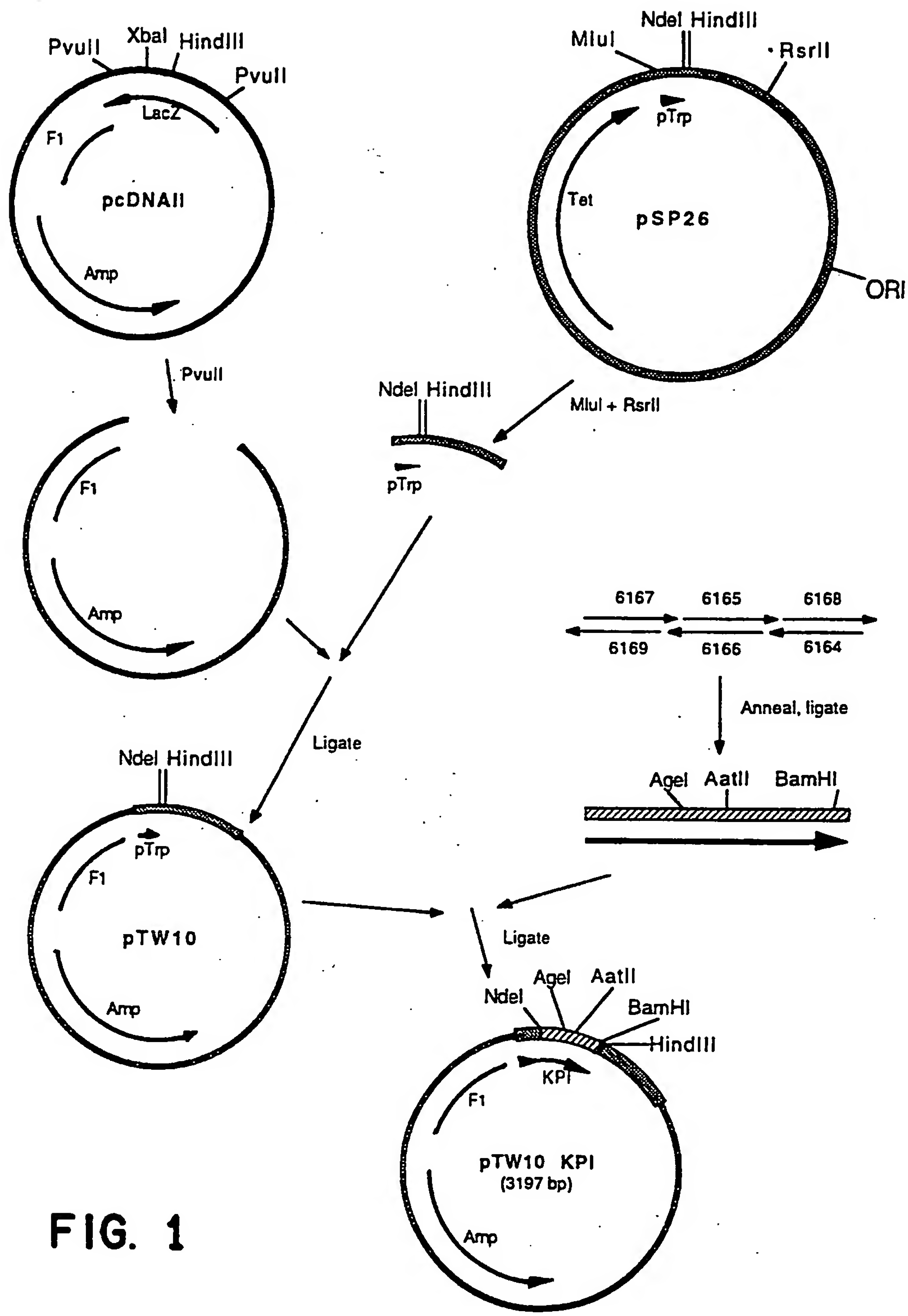
129. A protease inhibitor according to claim 126, wherein X^5 is Trp.

130. A protease inhibitor according to claim 128, wherein X^3 is Ala or Leu.

131. A protease inhibitor according to claim 130, wherein X^3 is Ala.

132. A protease inhibitor according to claim 130, wherein X^3 is Leu.

133. A protease inhibitor according to claim 129, wherein X^3 is Ala.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pSP26
ORI
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NdeI HindIII
MluI + RsrII
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Amp
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6169
6166
6164
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F1
pTW10
Amp
Ligate
NdeI
AgeI
AatII
BamHI
HindIII
KPI
F1
pTW10 KPI
(3197 bp)
Amp

FIG. 1

FIG. 2

```
Ndel
TATG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCT GTG ACA AAA
  AC TTT GTT TCG TGA TAA CGT GAC CGT GAG AAT GGC AAT GAC AAA TGG GGA CAC TGT TTT
 Met Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

      KPI                          Agel
GCC  GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG
CGG  CTC CAC ACG AGA CTT GTT CGA CTC TGG CCA GGC ACG GCA CGT TAC TAG AGG GCG ACC
Ala  Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp

        AatII
TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC
ATG AAA CTG CAG TGA CTT CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG
Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn

                                                         BamHI           HindIII
CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TA
GCA TTG TTG AAA CTG TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA
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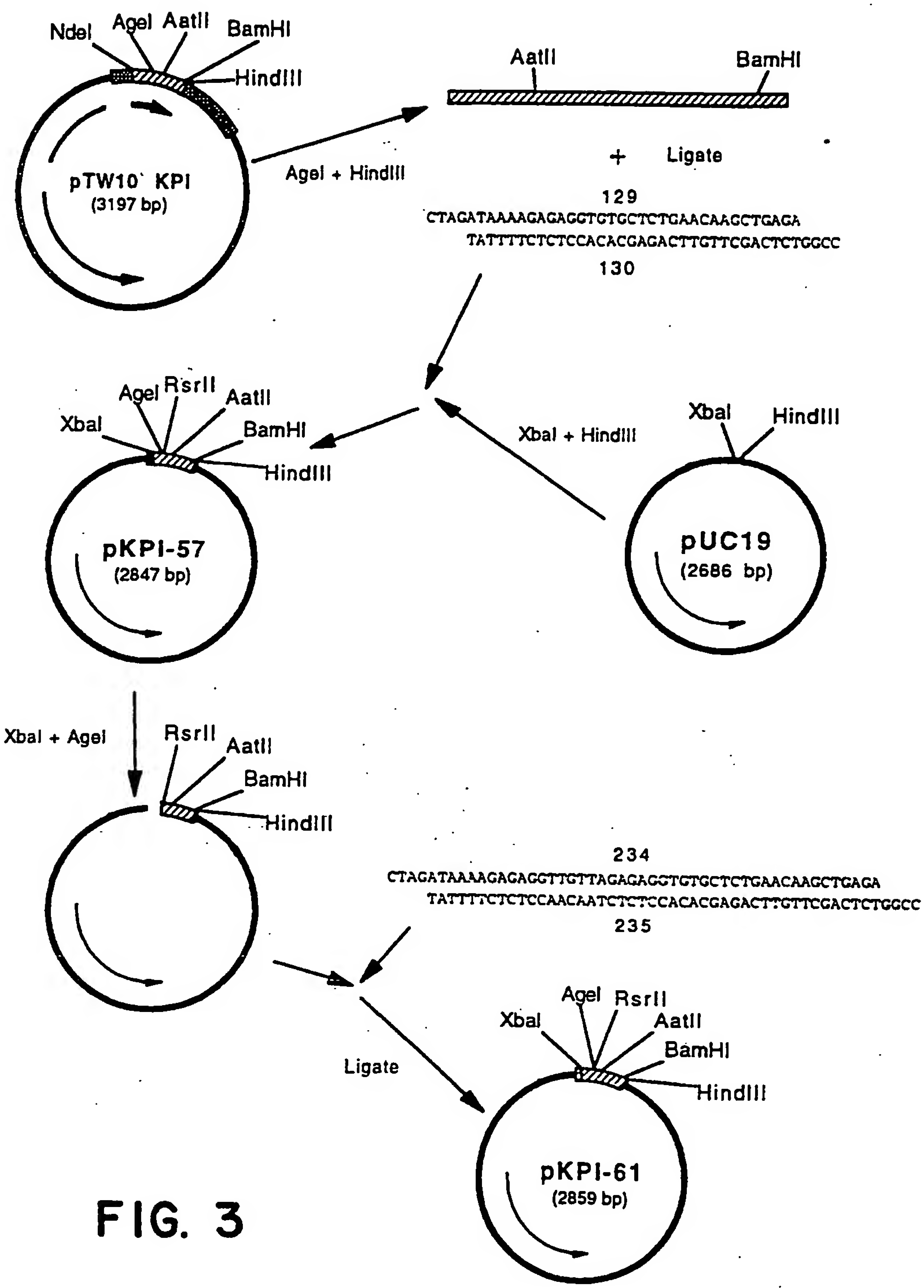
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AatII
BamHI
HindIII
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AatII
BamHI
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235
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AgeI
RsrII
AatII
BamHI
HindIII
pKPI-61
(2859 bp)

FIG. 3

FIG. 4

```
                              RsrII
Xbal      KPI (1-57)          AgeI
CTA GAT AAA AGA|GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT
     TA TTT TCT|CTC CAC ACG AGA CTT GTT CGA CTC TGG CCA GGC ACG GCA
▸Leu Asp Lys Arg|Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg

                         AatII
GCA ATG ATC TCC CGC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA
CGT TAC TAG AGG GCG ACC ATG AAA CTG CAG TGA CTT CCA TTC ACG CGA GGT
▸Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro

TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA GAG
AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG TGA CTT CTC
▸Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu

                  BamHI       HindIII
TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TA
ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA
▸Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 5

```
                                                                        RsrII
               KPI (-4-57)                                              AgeI
XbaI
CTA GAT AAA AGA|GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT
     TA TTT TCT|CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT CGA CTC TGG CCA
Leu Asp Lys Arg|Glu Val Val Arg Glu Val Cys Ser Glu Gln Ala Glu Thr Gly

                                            AatII
CCG TGC CGT GCA ATG ATC TCC CGC TGG TAC TTT GAC GTC ACT GAA GGT AAG TGC
GGC ACG GCA CGT TAC TAG AGG GCG ACC ATG AAA CTG CAG TGA CTT CCA TTC ACG
Pro Cys Arg Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu Gly Lys Cys

GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC ACT GAA
CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG TGA CTT
Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu

                            BamHI       HindIII
GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TA
CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA
Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
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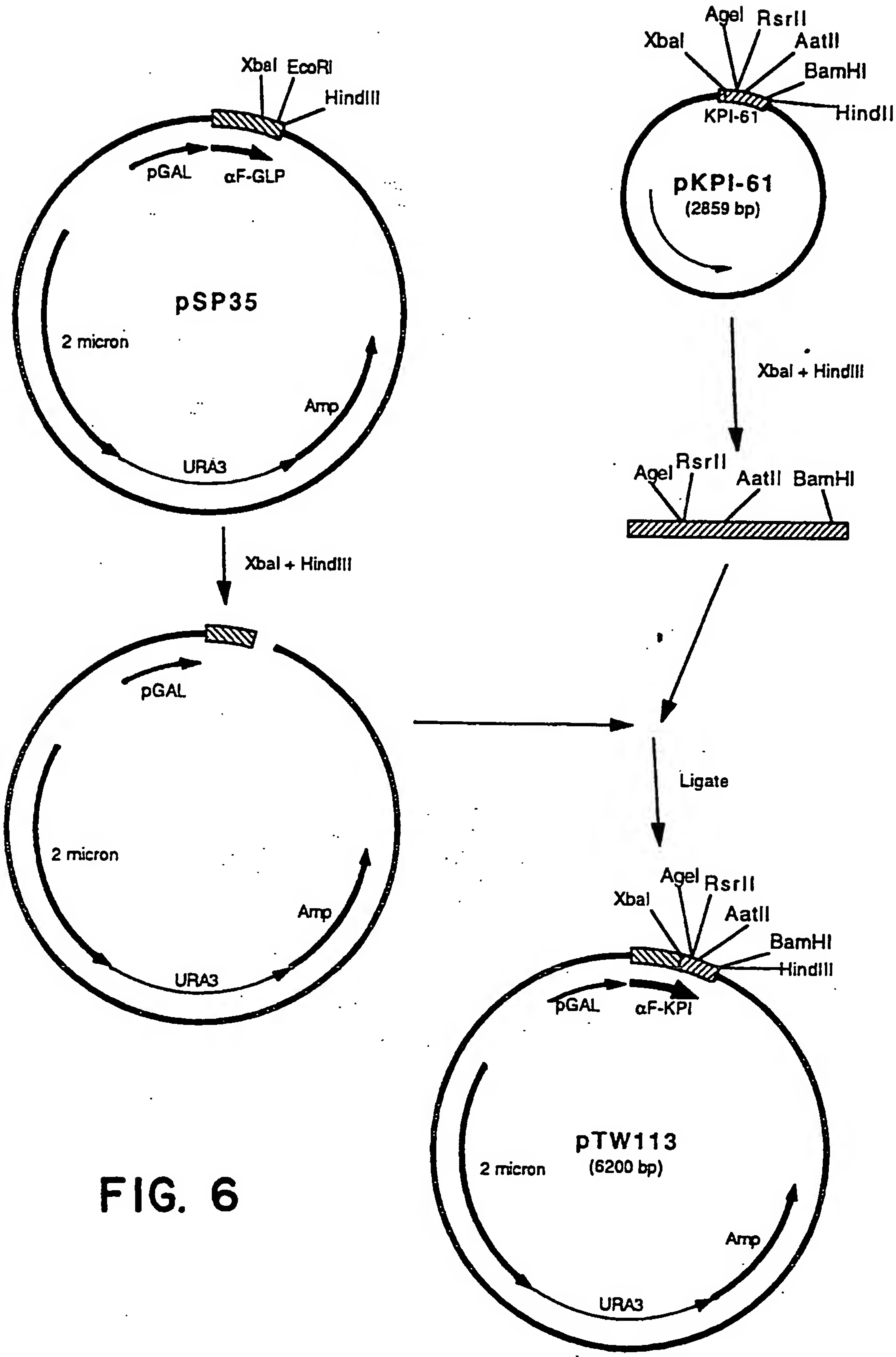
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EcoRI
HindIII
pGAL
αF-GLP
pSP35
2 micron
Amp
URA3
XbaI + HindIII
pGAL
2 micron
Amp
URA3
AgeI
RsrII
XbaI
AatII
BamHI
HindIII
KPI-61
pKPI-61
(2859 bp)
XbaI + HindIII
AgeI
RsrII
AatII
BamHI
Ligate
AgeI
RsrII
XbaI
AatII
BamHI
HindIII
pGAL
αF-KPI
pTW113
(6200 bp)
2 micron
Amp
URA3

FIG. 6

FIG. 7

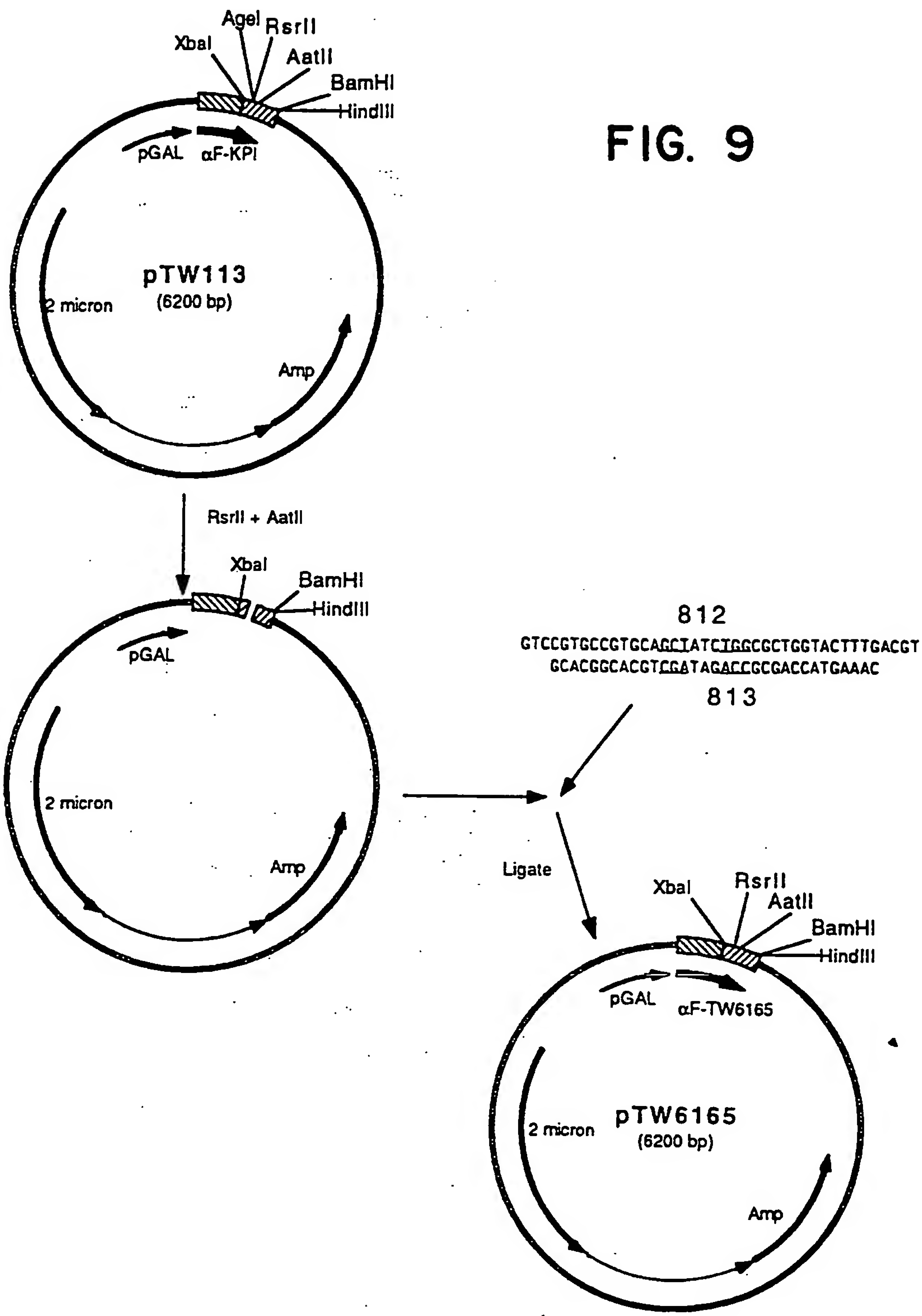
```
α-factor
 ──────►
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▸Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▸Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▸Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▸Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                                       KPI(-4-57)
                    XbaI                ──────►
GAA GAA GGG GTA TCT CTA GAT AAA AGA | GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT | CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▸Glu Glu Gly Val Ser Leu Asp Lys Arg | Glu Val Val Arg Glu Val Cys Ser Glu Gln

        RsrII
      AgeI                                                  AatII
GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT TAC TAG AGG GCG ACC ATG AAA CTG CAG TGA CTT
▸Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▸Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                    BamHI       HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▸Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 8

KPI(-4-57)

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Met -	Ile -	Ser -	Arg
8	9	10	11	12	13	14	15	16	17	18
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	Ala
19	20	21	22	23	24	25	26	27	28	29
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	Arg
30	31	32	33	34	35	36	37	38	39	40
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	Ala
41	42	43	44	45	46	47	48	49	50	51
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIG. 9


XbaI
AgeI
RsrII
AatII
BamHI
HindIII
pGAL
αF-KPI
pTW113
(6200 bp)
2 micron
Amp
RsrII + AatII
XbaI
BamHI
HindIII
pGAL
2 micron
Amp
812
GTCCGTGCCGTGCAGCTATCTGGCGCTGGTACTTTGACGT
GCACGGCACGTCGATAGACCGCGACCATGAAAC
813
Ligate
XbaI
RsrII
AatII
BamHI
HindIII
pGAL
αF-TW6165
pTW6165
(6200 bp)
2 micron
Amp

pTW 6165

FIG. 10

```
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▸Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▸Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▸Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▸Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                    XbaI                KPI(-4-57; M15A, S17W)
GAA GAA GGG GTA TCT CTA GAT AAA AGA | GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT | CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▸Glu Glu Gly Val Ser Leu Asp Lys Arg | Glu Val Val Arg Glu Val Cys Ser Glu Gln

        RsrII
        AgeI                                               AatII
GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TGG CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG ACC GCG ACC ATG AAA CTG CAG TGA CTT
▸Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Trp Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▸Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                     BamHI     HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▸Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 11

812
GTCCGTGCCGTGCAGCTATCTGGCGCTGGTACTTTGACGT
GCACGGCACGTCGATAGACCGCGACCATGAAAC
813

pTW6165 KPI(-4-57; M15A, S17F)

814
GTCCGTGCCGTGCAGCTATCTACCGCTGGTACTTTGACGT
GCACGGCACGTCGATAGATGGCGACCATGAAAC
815

pTW6166 KPI(-4-57; M15A, S17Y)

867
GTCCGTGCCGTGCATTGATCTTCCGCTGGTACTTTGACGT
GCACGGCACGTAACTAGAAGGCGACCATGAAAC
868

pTW6175 KPI(-4-57; M15L, S17F)

1493
GTCCGTGCCGTGCATTGATCTACCGCTGGTACTTTGACGT
GCACGGCACGTAACTAGATGGCGACCATGAAAC
1494

pBG028 KPI(-4-57; M15L, S17Y)

925
GTCCGTGCCGTGCAATGCACTTCCGCTGGTACTTTGACGT
GCACGGCACGTTACGTGAAGGCGACCATGAAAC
926

pTW6183 KPI(-4-57; I16H, S17F)

927
GTCCGTGCCGTGCAATGCACTACCGCTGGTACTTTGACGT
GCACGGCACGTTACGTGATGGCGACCATGAAAC
928

pTW6184 KPI(-4-57; I16H, S17Y)

929
GTCCGTGCCGTGCAATGCACTGGCGCTGGTACTTTGACGT
GCACGGCACGTTACGTGACCGCGACCATGAAAC
930

pTW6185 KPI(-4-57; I16H, S17W)

863
GTCCGTGCCGTGCAGCTCACTCCCGCTGGTACTTTGACGT
GCACGGCACGTCGAGTGAGGGCGACCATGAAAC
864

pTW6173 KPI(-4-57; M15A, I16H)

865
GTCCGTGCCGTGCATTGCACTCCCGCTGGTACTTTGACGT
GCACGGCACGTAACGTGAGGGCGACCATGAAAC
866

pTW6174 KPI(-4-57; M15L, I16H)

pTW 6166

FIG. 12

```
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                XbaI              KPI(-4-57; M15A, S17Y)
GAA GAA GGG GTA TCT CTA GAT AAA AGA|GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT|CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg|Glu Val Val Arg Glu Val Cys Ser Glu Gln

         RsrII
      AgeI                                                  AatII
GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TAC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                        BamHI       HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 13

```
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                     XbaI                   KPI(-4-57; M15L, S17F)
GAA GAA GGG GTA TCT CTA GAT AAA AGA | GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT | CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg | Glu Val Val Arg Glu Val Cys Ser Glu Gln

          RsrII
        AgeI                                                 AatII
GCT GAG ACC GGT CCG TGC CGT GCA TTG ATC TTC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT AAC TAG AAG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Leu Ile Phe Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                     BamHI       HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 14

```
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                            XbaI              KPI(-4-57; M15L, S17Y)
GAA GAA GGG GTA TCT CTA GAT AAA AGA | GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT | CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg | Glu Val Val Arg Glu Val Cys Ser Glu Gln

         RsrII
       AgeI                                                     AatII
GCT GAG ACC GGT CCG TGC CGT GCA TTG ATC TAC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT AAC TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Leu Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                    BamHI           HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 15

α-factor

```
 ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
 TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▸Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

 GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
 CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▸Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

 ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
 TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▸Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

 AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
 TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▸Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                                         KPI(-4-57; I16H, S17F)
                         XbaI
 GAA GAA GGG GTA TCT CTA GAT AAA AGA|GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
 CTT CTT CCC CAT AGA GAT CTA TTT TCT|CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▸Glu Glu Gly Val Ser Leu Asp Lys Arg|Glu Val Val Arg Glu Val Cys Ser Glu Gln

         RsrII
         AgeI                                                   AatII
 GCT GAG ACC GGT CCG TGC CGT GCA ATG CAC TTC CGC TGG TAC TTT GAC GTC ACT GAA
 CGA CTC TGG CCA GGC ACG GCA CGT TAC GTG AAG GCG ACC ATG AAA CTG CAG TGA CTT
▸Ala Glu Thr Gly Pro Cys Arg Ala Met His Phe Arg Trp Tyr Phe Asp Val Thr Glu

 GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
 CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▸Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                         BamHI           HindIII
 ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
 TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▸Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 16

```
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                    XbaI                 KPI(-4-57; I16H, S17Y)
GAA GAA GGG GTA TCT CTA GAT AAA AGA | GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT | CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg | Glu Val Val Arg Glu Val Cys Ser Glu Gln

           RsrII
        AgeI                                                 AatII
GCT GAG ACC GGT CCG TGC CGT GCA ATG CAC TAC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT TAC GTG ATG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Met His Tyr Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                         BamHI       HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 17

```
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                        XbaI                KPI(-4-57; I16H, S17W)
GAA GAA GGG GTA TCT CTA GAT AAA AGA | GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT | CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg | Glu Val Val Arg Glu Val Cys Ser Glu Gln

            RsrII
         AgeI                                                  AatII
GCT GAG ACC GGT CCG TGC CGT GCA ATG CAC TGG CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT TAC GTG ACC GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Met His Trp Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                       BamHI      HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 18

```
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                    XbaI                KPI(-4-57; M15A, I16H)
GAA GAA GGG GTA TCT CTA GAT AAA AGA | GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT | CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg | Glu Val Val Arg Glu Val Cys Ser Glu Gln

          RsrII
        AgeI                                                   AatII
GCT GAG ACC GGT CCG TGC CGT GCA GCT CAC TCC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT CGA GTG AGG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Ala His Ser Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                     BamHI           HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 19

```
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▸Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▸Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▸Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▸Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                                        KPI(-4-57; M15L, I16H)
                   XbaI
GAA GAA GGG GTA TCT CTA GAT AAA AGA|GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT|CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▸Glu Glu Gly Val Ser Leu Asp Lys Arg|Glu Val Val Arg Glu Val Cys Ser Glu Gln

        RsrII
        AgeI                                                   AatII
GCT GAG ACC GGT CCG TGC CGT GCA TTG CAC TCC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT AAC GTG AGG GCG ACC ATG AAA CTG CAG TGA CTT
▸Ala Glu Thr Gly Pro Cys Arg Ala Leu His Ser Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▸Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                        BamHI       HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▸Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 20

KPI(-4-57; M15A, S17W) TW6165

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Ala -	Ile -	Trp -	Arg
8	9	10	11	12	13	14	15	16	17	18
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	Ala
19	20	21	22	23	24	25	26	27	28	29
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	Arg
30	31	32	33	34	35	36	37	38	39	40
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	Ala
41	42	43	44	45	46	47	48	49	50	51
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIG. 21

KPI(-4-57; M15A, S17Y) TW6166

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Ala -	Ile -	Tyr -	Arg
8	9	10	11	12	13	14	15	16	17	18
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	Ala
19	20	21	22	23	24	25	26	27	28	29
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	Arg
30	31	32	33	34	35	36	37	38	39	40
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	Ala
41	42	43	44	45	46	47	48	49	50	51
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIG. 22

KPI(-4-57; M15L, S17F) TW6175

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	<u>Leu</u> -	Ile -	<u>Phe</u> -	Arg
8	9	10	11	12	13	14	15	16	17	18
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	Ala
19	20	21	22	23	24	25	26	27	28	29
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	Arg
30	31	32	33	34	35	36	37	38	39	40
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	Ala
41	42	43	44	45	46	47	48	49	50	51
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIG. 23

KPI(-4-57; M15L, S17Y) BG028

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Leu -	Ile -	Tyr -	Arg
8	9	10	11	12	13	14	15	16	17	18
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	Ala
19	20	21	22	23	24	25	26	27	28	29
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	Arg
30	31	32	33	34	35	36	37	38	39	40
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	Ala
41	42	43	44	45	46	47	48	49	50	51
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIG. 24

KPI(-4-57; I16H, S17F) TW6183

Glu - Val - Val - Arg - Glu - Val - Cys - Ser - Glu - Gln - Ala
-4 -3 -2 -1 1 2 3 4 5 6 7

Glu - Thr - Gly - Pro - Cys - Arg - Ala - Met - <u>His</u> - <u>Phe</u> - Arg
8 9 10 11 12 13 14 15 16 17 18

Trp - Tyr - Phe - Asp - Val - Thr - Glu - Gly - Lys - Cys - Ala
19 20 21 22 23 24 25 26 27 28 29

Pro - Phe - Phe - Tyr - Gly - Gly - Cys - Gly - Gly - Asn - Arg
30 31 32 33 34 35 36 37 38 39 40

Asn - Asn - Phe - Asp - Thr - Glu - Glu - Tyr - Cys - Met - Ala
41 42 43 44 45 46 47 48 49 50 51

Val - Cys - Gly - Ser - Ala - Ile
52 53 54 55 56 57

FIG. 25

KPI(-4-57; I16H, S17Y) TW6184

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Met -	His -	Tyr -	Arg
8	9	10	11	12	13	14	15	16	17	18
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	Ala
19	20	21	22	23	24	25	26	27	28	29
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	Arg
30	31	32	33	34	35	36	37	38	39	40
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	Ala
41	42	43	44	45	46	47	48	49	50	51
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIG. 26

KPI(-4-57; I16H, S17W) TW6185

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Met -	His -	Trp -	Arg
8	9	10	11	12	13	14	15	16	17	18
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	Ala
19	20	21	22	23	24	25	26	27	28	29
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	Arg
30	31	32	33	34	35	36	37	38	39	40
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	Ala
41	42	43	44	45	46	47	48	49	50	51
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIG. 27

KPI(-4-57; M15A, S17F) DD185

Glu	-	Val	-	Val	-	Arg	-	Glu	-	Val	-	Cys	-	Ser	-	Glu	-	Gln	-	Ala
-4		-3		-2		-1		1		2		3		4		5		6		7
Glu	-	Thr	-	Gly	-	Pro	-	Cys	-	Arg	-	Ala	-	Ala	-	Ile	-	Phe	-	Arg
8		9		10		11		12		13		14		15		16		17		18
Trp	-	Tyr	-	Phe	-	Asp	-	Val	-	Thr	-	Glu	-	Gly	-	Lys	-	Cys	-	Ala
19		20		21		22		23		24		25		26		27		28		29
Pro	-	Phe	-	Phe	-	Tyr	-	Gly	-	Gly	-	Cys	-	Gly	-	Gly	-	Asn	-	Arg
30		31		32		33		34		35		36		37		38		39		40
Asn	-	Asn	-	Phe	-	Asp	-	Thr	-	Glu	-	Glu	-	Tyr	-	Cys	-	Met	-	Ala
41		42		43		44		45		46		47		48		49		50		51
Val	-	Cys	-	Gly	-	Ser	-	Ala	-	Ile										
52		53		54		55		56		57										

FIG. 28

KPI(-4-57; M15A, I16H) TW6173

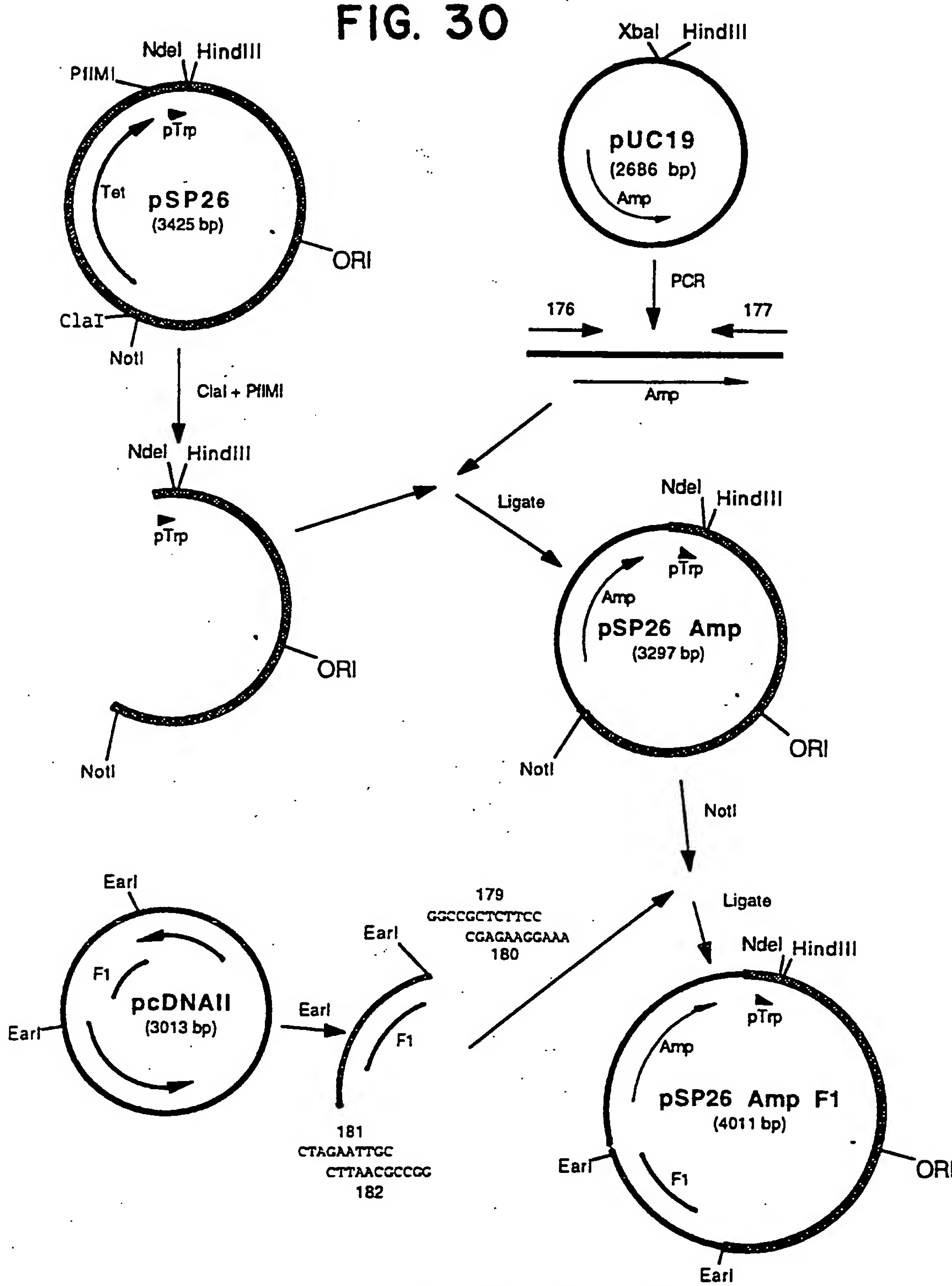
Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	Ala -	His -	Ser -	Arg
8	9	10	11	12	13	14	15	16	17	18
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	Ala
19	20	21	22	23	24	25	26	27	28	29
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	Arg
30	31	32	33	34	35	36	37	38	39	40
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	Ala
41	42	43	44	45	46	47	48	49	50	51
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIG. 29

KPI(-4-57; M15L, I16H) TW6174

Glu -	Val -	Val -	Arg -	Glu -	Val -	Cys -	Ser -	Glu -	Gln -	Ala
-4	-3	-2	-1	1	2	3	4	5	6	7
Glu -	Thr -	Gly -	Pro -	Cys -	Arg -	Ala -	<u>Leu</u> -	<u>His</u> -	Ser -	Arg
8	9	10	11	12	13	14	15	16	17	18
Trp -	Tyr -	Phe -	Asp -	Val -	Thr -	Glu -	Gly -	Lys -	Cys -	Ala
19	20	21	22	23	24	25	26	27	28	29
Pro -	Phe -	Phe -	Tyr -	Gly -	Gly -	Cys -	Gly -	Gly -	Asn -	Arg
30	31	32	33	34	35	36	37	38	39	40
Asn -	Asn -	Phe -	Asp -	Thr -	Glu -	Glu -	Tyr -	Cys -	Met -	Ala
41	42	43	44	45	46	47	48	49	50	51
Val -	Cys -	Gly -	Ser -	Ala -	Ile					
52	53	54	55	56	57					

FIG. 30



PflMI
NdeI
HindIII
pTrp
Tet
pSP26
(3425 bp)
ORI
ClaI
NotI
ClaI + PflMI
XbaI
HindIII
pUC19
(2686 bp)
Amp
PCR
176
177
Amp
NdeI
HindIII
pTrp
ORI
NotI
Ligate
NdeI
HindIII
pTrp
Amp
pSP26 Amp
(3297 bp)
ORI
NotI
NotI
Ligate
EarI
F1
pcDNAII
(3013 bp)
EarI
EarI
EarI
F1
179
GGCCGCTCTTCC
CGAGAAGGAAA
180
181
CTAGAATTGC
CTTAACGCCCGG
182
NdeI
HindIII
pTrp
Amp
pSP26 Amp F1
(4011 bp)
EarI
F1
ORI
EarI

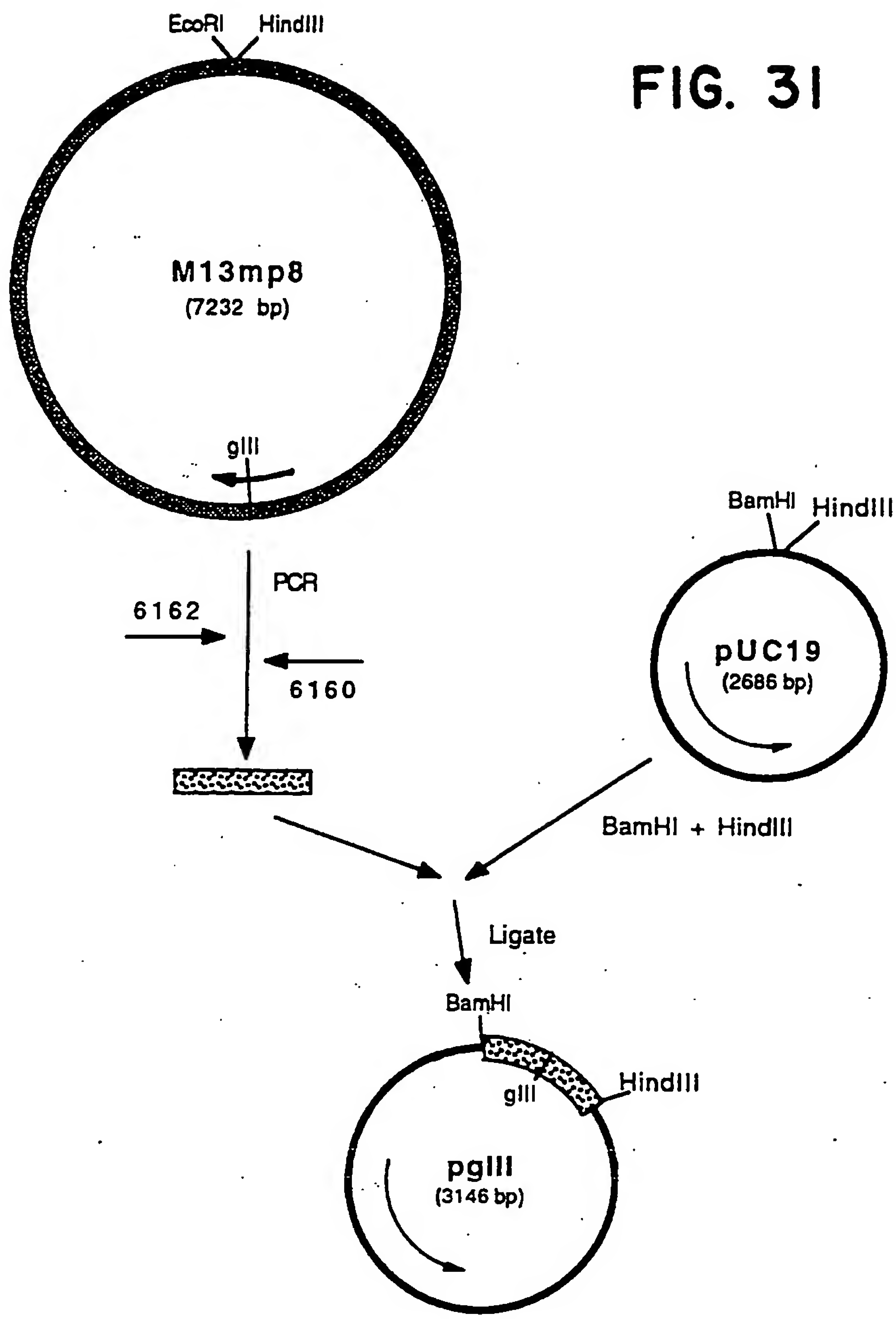

EcoRI
HindIII
M13mp8
(7232 bp)
gIII
PCR
6162
6160
BamHI
HindIII
pUC19
(2686 bp)
BamHI + HindIII
Ligate
BamHI
HindIII
gIII
pgIII
(3146 bp)

FIG. 31

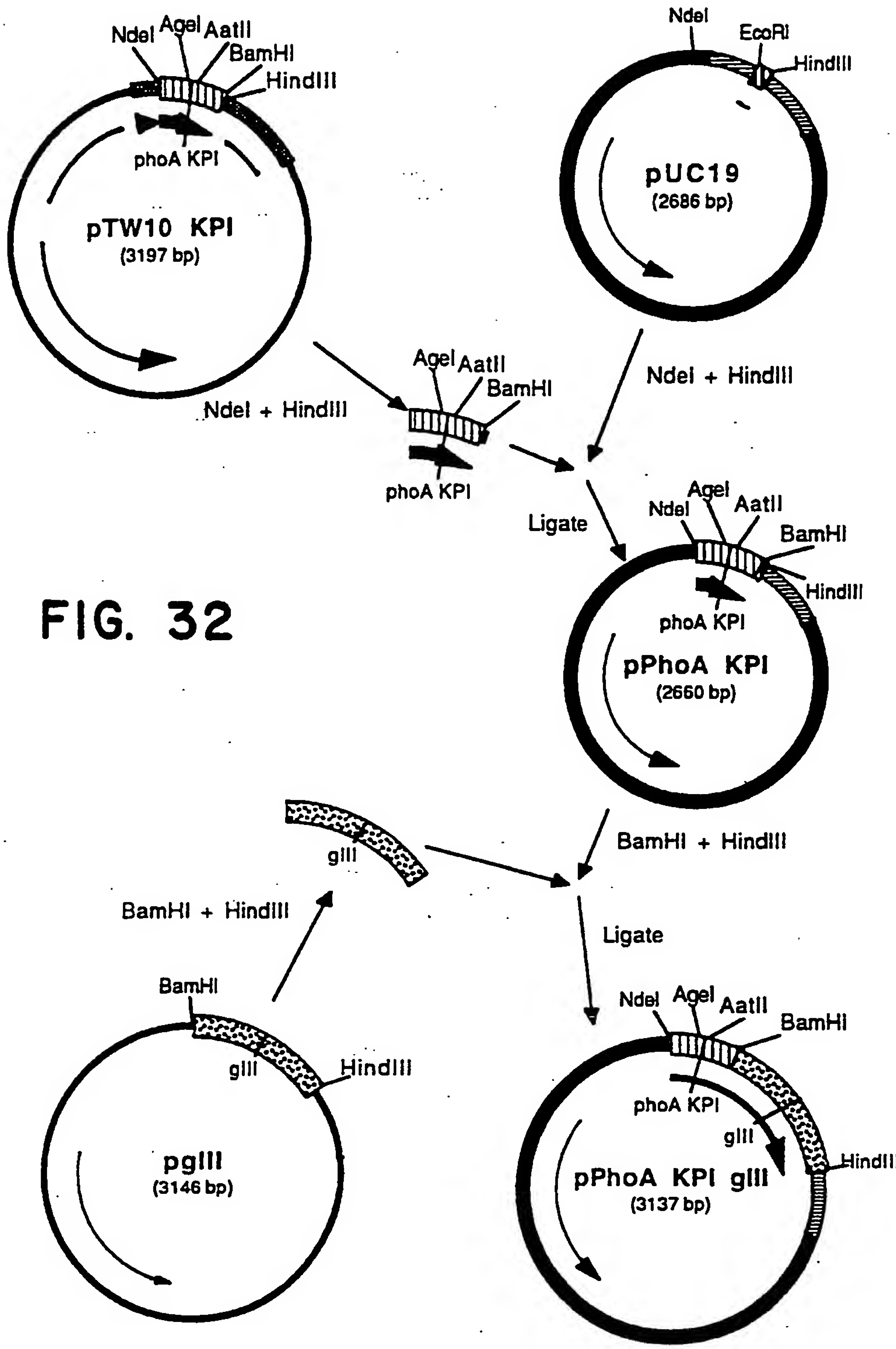
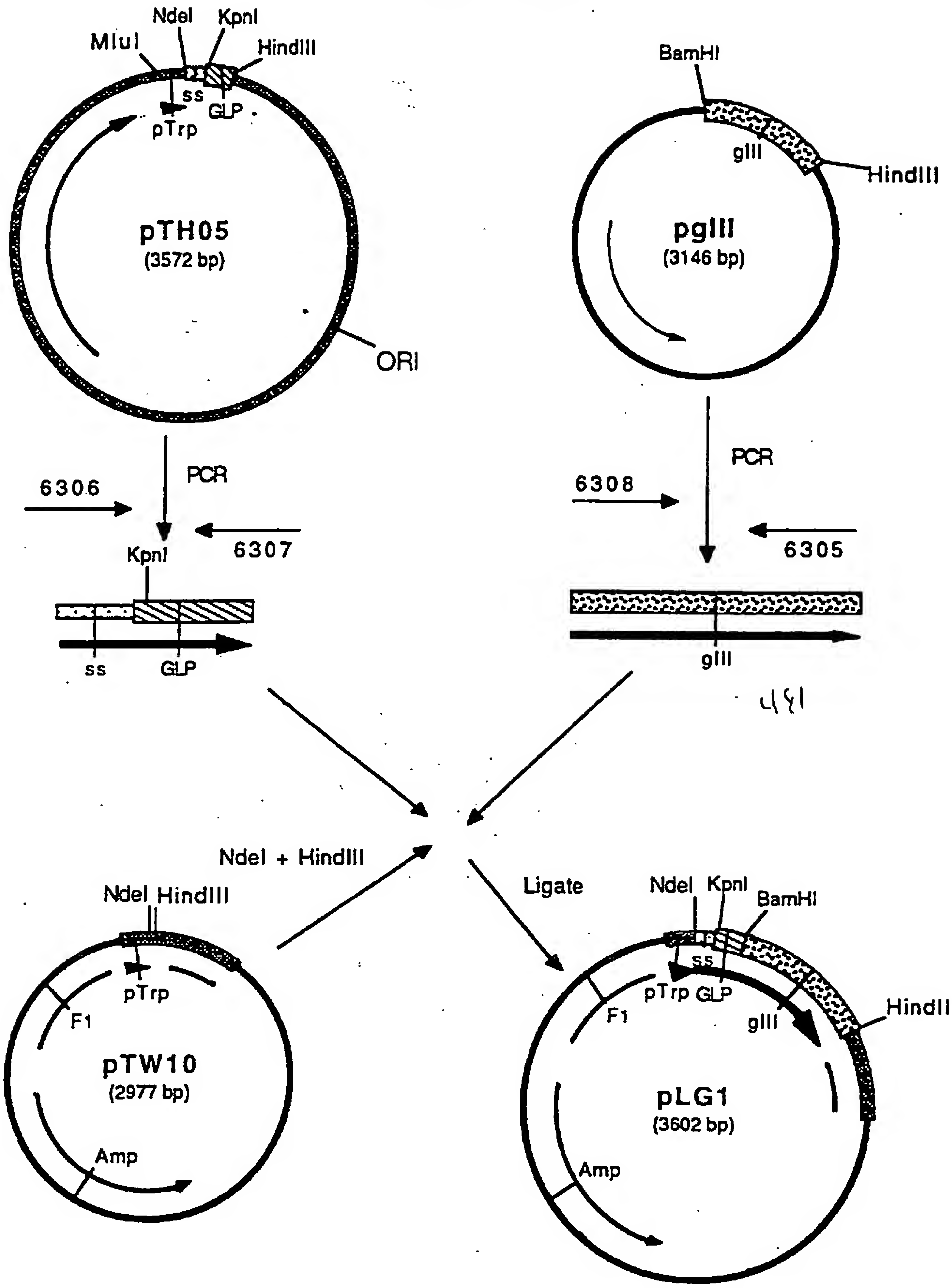

NdeI
AgeI
AatII
BamHI
HindIII
phoA KPI
pTW10 KPI
(3197 bp)
NdeI
EcoRI
HindIII
pUC19
(2686 bp)
NdeI + HindIII
AgeI
AatII
BamHI
phoA KPI
NdeI + HindIII
Ligate
NdeI
AgeI
AatII
BamHI
HindIII
phoA KPI
pPhoA KPI
(2660 bp)
gIII
BamHI + HindIII
BamHI + HindIII
Ligate
BamHI
gIII
HindIII
pgIII
(3146 bp)
NdeI
AgeI
AatII
BamHI
phoA KPI
gIII
HindIII
pPhoA KPI gIII
(3137 bp)

FIG. 32

FIG. 33


MluI
NdeI
KpnI
HindIII
ss
GLP
pTrp
pTH05
(3572 bp)
ORI
BamHI
gIII
HindIII
pgIII
(3146 bp)
6306
PCR
6307
KpnI
ss
GLP
6308
PCR
6305
gIII
NdeI + HindIII
NdeI HindIII
pTrp
F1
pTW10
(2977 bp)
Amp
Ligate
NdeI
KpnI
BamHI
ss
pTrp
GLP
F1
gIII
HindIII
pLG1
(3602 bp)
Amp

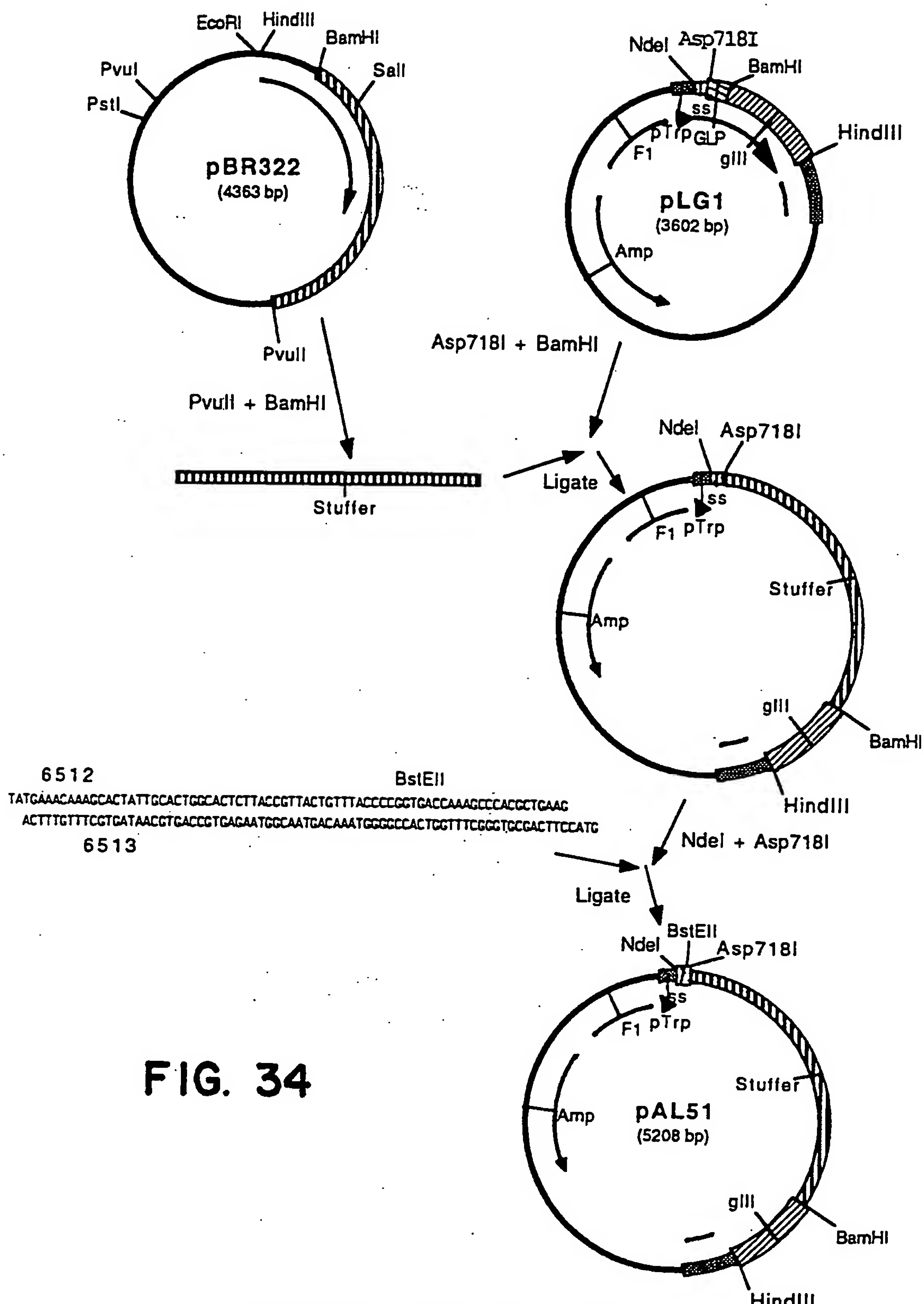
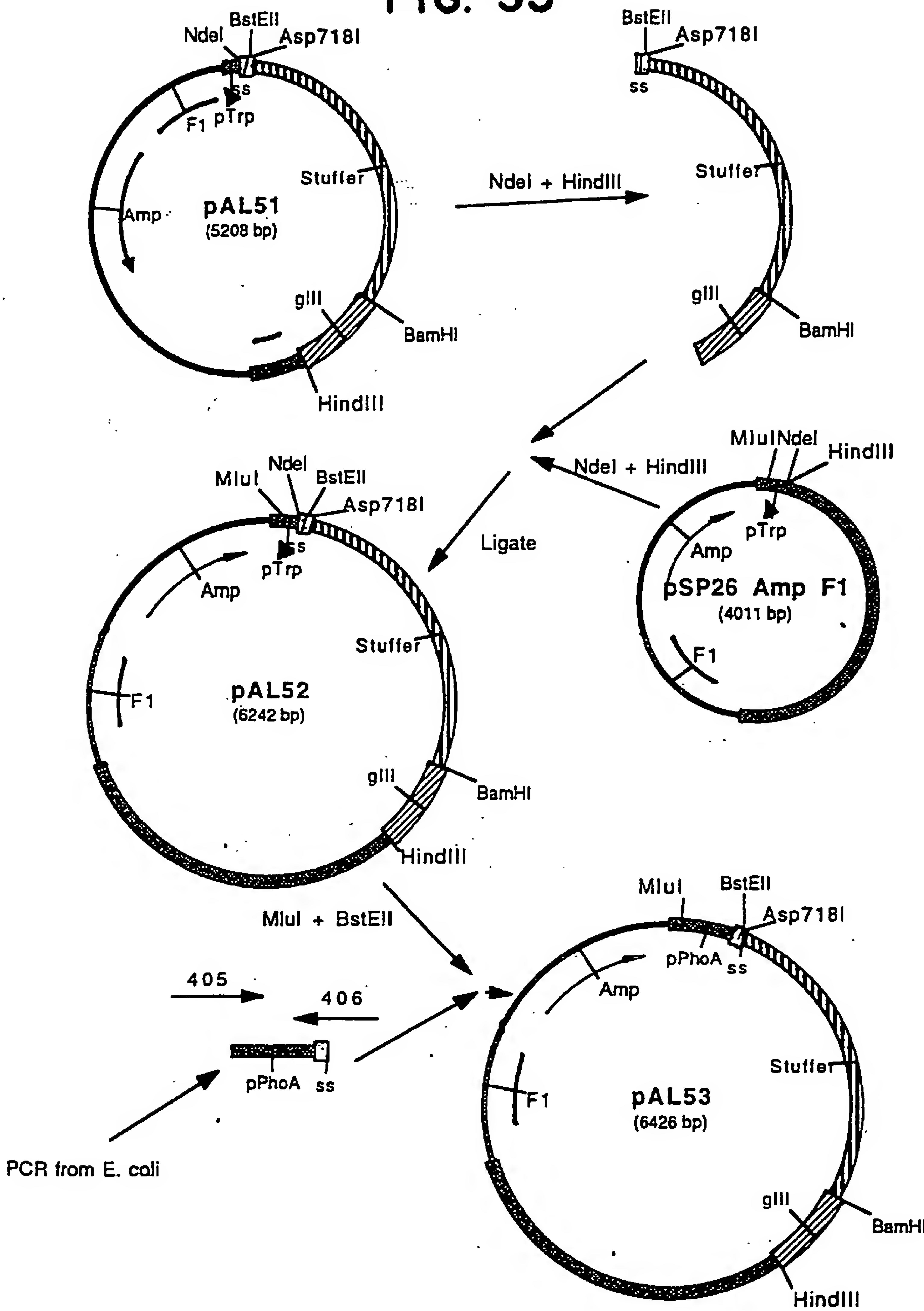

EcoRI
HindIII
BamHI
PvuI
SalI
PstI
pBR322
(4363 bp)
PvuII
PvuII + BamHI
Stuffer
NdeI
Asp718I
BamHI
ss
pTrp
GLP
F1
gIII
HindIII
pLG1
(3602 bp)
Amp
Asp718I + BamHI
Ligate
NdeI
Asp718I
ss
F1
pTrp
Stuffer
Amp
gIII
BamHI
HindIII
6512
BstEII
TATGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCCCGGTGACCAAAGCCCACGCTGAAC
ACTTTGTTTCGTGATAACGTGACCGTGAGAATGGCAATGACAAATGGGGCCACTGGTTTCGGGTGCGACTTCCATG
6513
NdeI + Asp718I
Ligate
BstEII
NdeI
Asp718I
ss
F1
pTrp
Stuffer
Amp
pAL51
(5208 bp)
gIII
BamHI
HindIII

FIG. 34

FIG. 35



NdeI
BstEII
Asp718I
ss
F1
pTrp
Amp
pAL51
(5208 bp)
Stuffer
gIII
BamHI
HindIII
NdeI + HindIII
BstEII
Asp718I
ss
Stuffer
gIII
BamHI
MluI
NdeI
BstEII
Asp718I
ss
pTrp
Amp
F1
pAL52
(6242 bp)
Stuffer
gIII
BamHI
HindIII
Ligate
NdeI + HindIII
MluI NdeI
HindIII
pTrp
Amp
pSP26 Amp F1
(4011 bp)
F1
MluI + BstEII
405
406
pPhoA
ss
PCR from E. coli
MluI
BstEII
Asp718I
pPhoA
ss
Amp
F1
pAL53
(6426 bp)
Stuffer
gIII
BamHI
HindIII

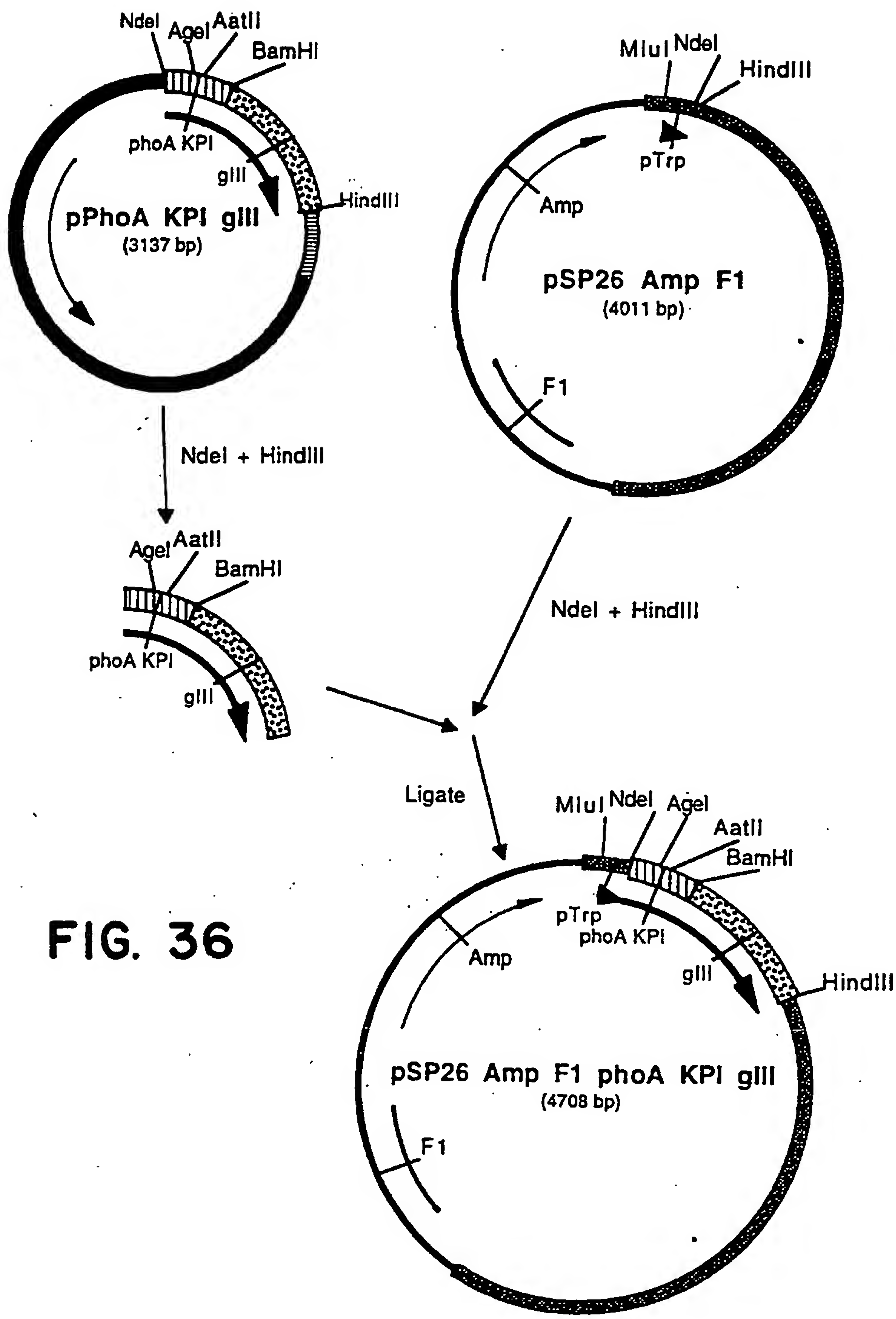

NdeI
AgeI
AatII
BamHI
phoA KPI
gIII
HindIII
pPhoA KPI gIII
(3137 bp)
MluI
NdeI
HindIII
pTrp
Amp
pSP26 Amp F1
(4011 bp)
F1
NdeI + HindIII
AgeI
AatII
BamHI
phoA KPI
gIII
NdeI + HindIII
Ligate
MluI
NdeI
AgeI
AatII
BamHI
pTrp
phoA KPI
Amp
gIII
HindIII
pSP26 Amp F1 phoA KPI gIII
(4708 bp)
F1

FIG. 36

FIG. 37

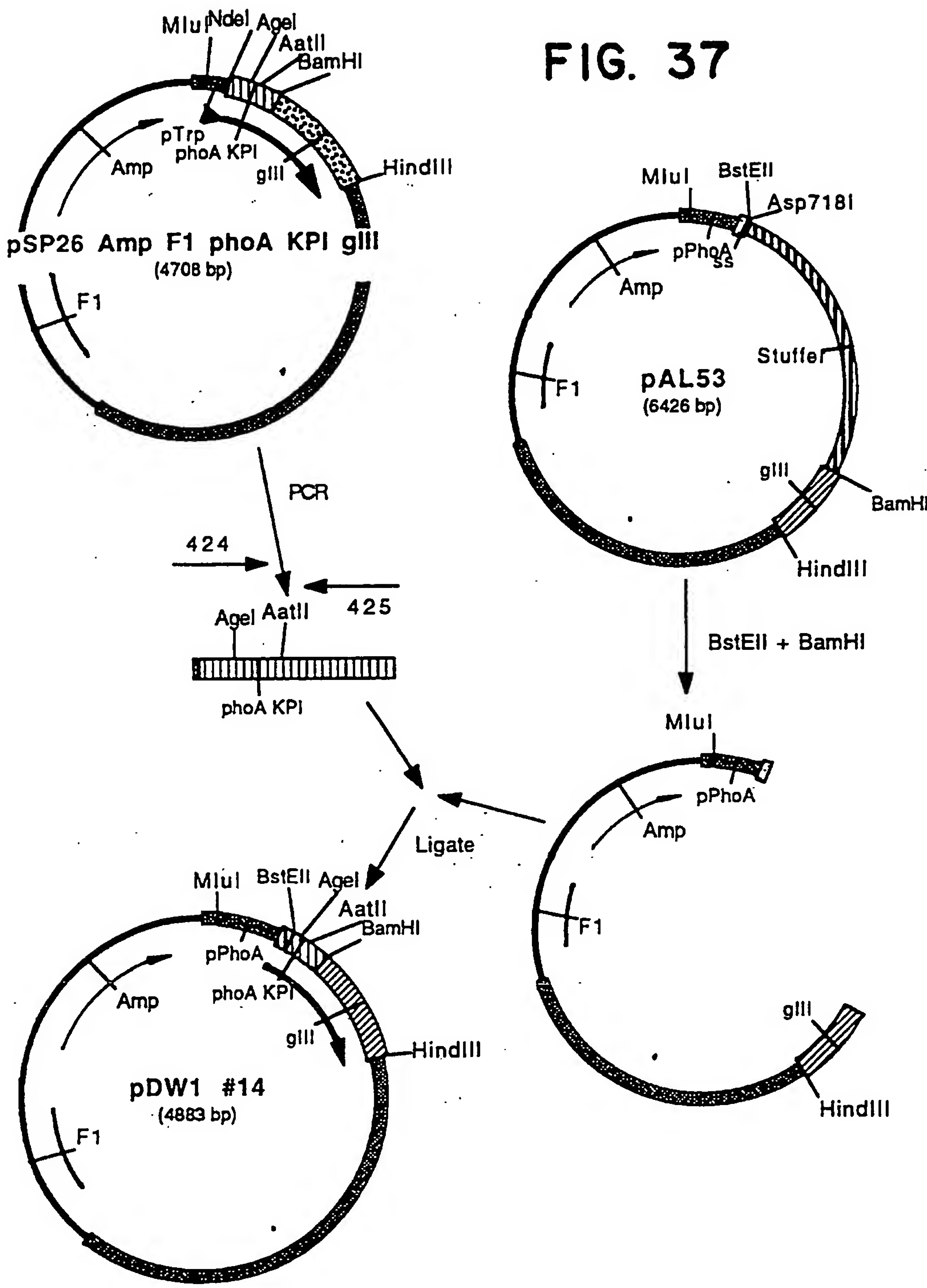

MluI
NdeI
AgeI
AatII
BamHI
pTrp
phoA KPI
Amp
gIII
HindIII
pSP26 Amp F1 phoA KPI gIII
(4708 bp)
F1
MluI
BstEII
Asp718I
pPhoA
ss
Amp
Stuffer
F1
pAL53
(6426 bp)
gIII
BamHI
HindIII
PCR
424
425
AgeI
AatII
phoA KPI
BstEII + BamHI
MluI
pPhoA
Amp
Ligate
F1
gIII
HindIII
MluI
BstEII
AgeI
AatII
BamHI
pPhoA
Amp
phoA KPI
gIII
HindIII
pDW1 #14
(4883 bp)
F1

FIG. 38

phoA signal → BstEII

GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

KPI (1-55) → AgeI

GCC | GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA ATG ATC TCC CGC TGG
Ala | Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Met Ile Ser Arg Trp

AatII

TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC
Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn

BamHI gIII →

CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC | GGT GGT GGC TCT
Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser | Gly Gly Gly Ser

GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA
Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu

AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT
Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr

GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT
Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn

GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT
Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp

AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA
Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu

TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA
Cys Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys

ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA
Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val

TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser •••

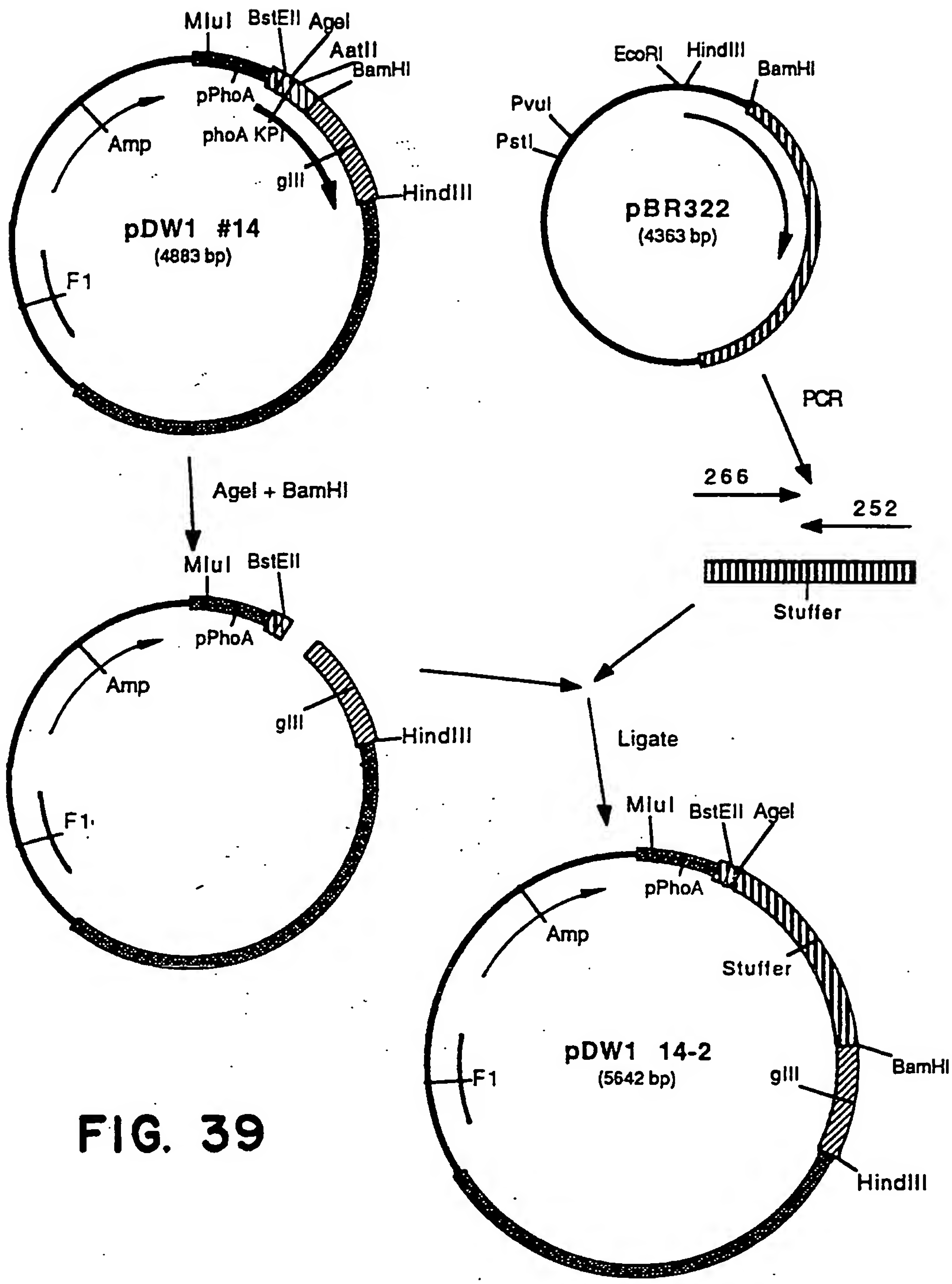

MluI
BstEII
AgeI
AatII
BamHI
pPhoA
phoA KPI
Amp
gIII
HindIII
pDW1 #14
(4883 bp)
F1
EcoRI
HindIII
BamHI
PvuI
PstI
pBR322
(4363 bp)
PCR
266
252
Stuffer
AgeI + BamHI
MluI
BstEII
pPhoA
Amp
gIII
HindIII
F1
Ligate
MluI
BstEII
AgeI
pPhoA
Amp
Stuffer
pDW1 14-2
(5642 bp)
gIII
BamHI
F1
HindIII

FIG. 39

FIG. 40

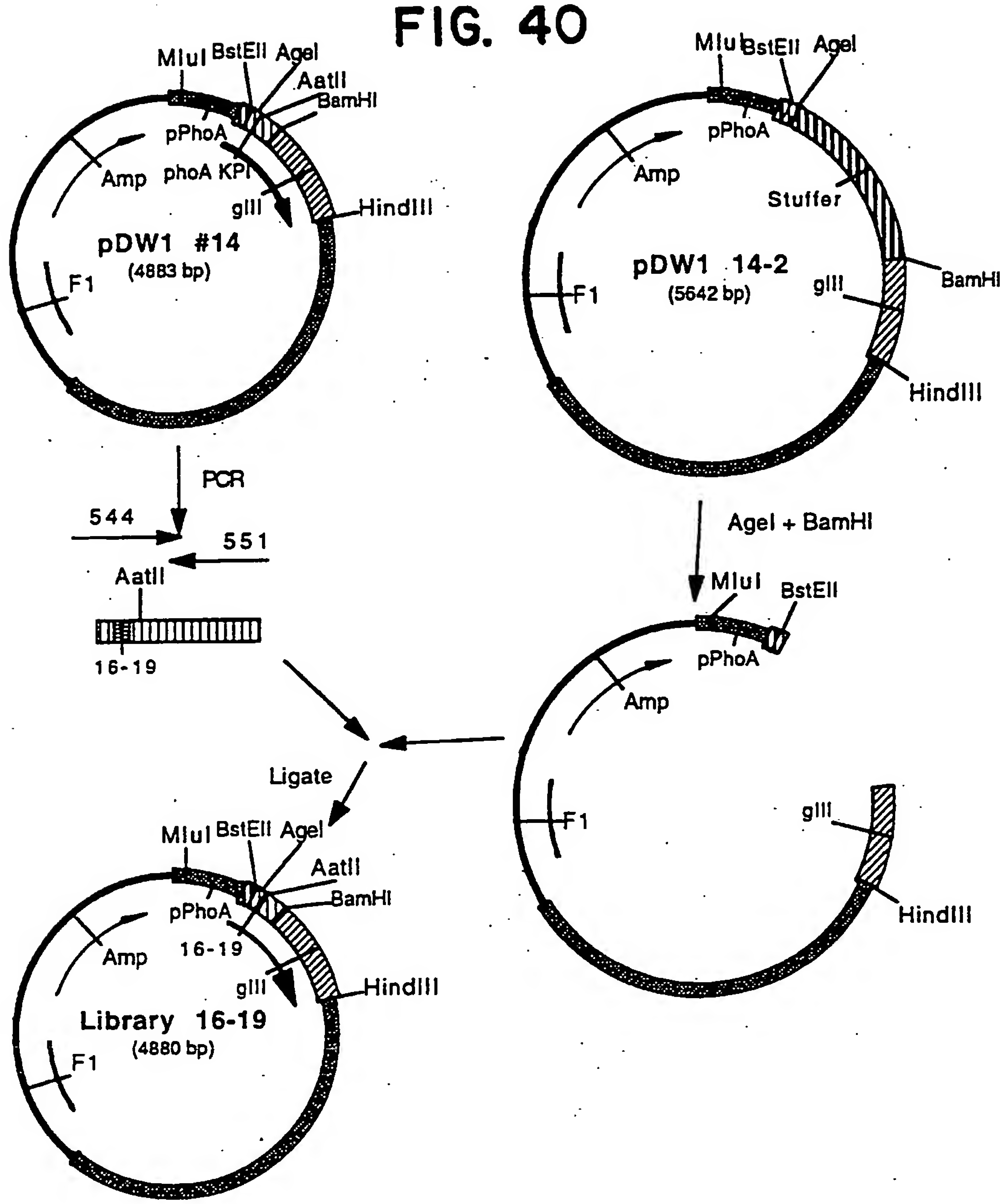

MluI
BstEII
AgeI
AatII
BamHI
pPhoA
Amp
phoA KPI
gIII
HindIII
pDW1 #14
(4883 bp)
F1
Stuffer
BamHI
pDW1 14-2
(5642 bp)
HindIII
PCR
544
551
AatII
16-19
AgeI + BamHI
Ligate
Library 16-19
(4880 bp)

FIG. 41

```
phoA signal
                                                                      BstEII
GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

    KPI (1-55; 16-19)               AgeI                   16-19
GCC GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT NNS NNS NNS NNS TGG TAC
Ala Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg ??? ??? ??? ??? Trp Tyr

    AatII
TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT
Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg

                                                         BamHI   gIII
AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GGT GGT GGC TCT GGT
Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Gly Gly Gly Ser Gly

TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA AAT
Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu Asn

GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT GAT
Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr Asp

TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT GGT
Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn Gly

                                                         gIII
GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT AAT
Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp Asn

TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA TGT
Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu Cys

CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA ATA
Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys Ile

AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA TTT
Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val Phe

TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser •••
```

FIG. 42

phoA signal

BstEII

GTG AAA CAA AGC ACT ATT GCA CTG GCA CTC TTA CCG TTA CTG TTT ACC CCG GTG ACC AAA
▸Val Lys Gln Ser Thr Ile Ala Leu Ala Leu Leu Pro Leu Leu Phe Thr Pro Val Thr Lys

KPI (1-55; M15A, S17F)

AgeI

GCC | GAG GTG TGC TCT GAA CAA GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TTC CGC TGG
▸Ala | Glu Val Cys Ser Glu Gln Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Phe Arg Trp

AatII

TAC TTT GAC GTC ACT GAA GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC
▸Tyr Phe Asp Val Thr Glu Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn

BamHI gIII

CGT AAC AAC TTT GAC ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC | GGT GGT GGC TCT
▸Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser | Gly Gly Gly Ser

GGT TCC GGT GAT TTT GAT TAT GAA AAG ATG GCA AAC GCT AAT AAG GGG GCT ATG ACC GAA
▸Gly Ser Gly Asp Phe Asp Tyr Glu Lys Met Ala Asn Ala Asn Lys Gly Ala Met Thr Glu

AAT GCC GAT GAA AAC GCG CTA CAG TCT GAC GCT AAA GGC AAA CTT GAT TCT GTC GCT ACT
▸Asn Ala Asp Glu Asn Ala Leu Gln Ser Asp Ala Lys Gly Lys Leu Asp Ser Val Ala Thr

GAT TAC GGT GCT GCT ATC GAT GGT TTC ATT GGT GAC GTT TCC GGC CTT GCT AAT GGT AAT
▸Asp Tyr Gly Ala Ala Ile Asp Gly Phe Ile Gly Asp Val Ser Gly Leu Ala Asn Gly Asn

GGT GCT ACT GGT GAT TTT GCT GGC TCT AAT TCC CAA ATG GCT CAA GTC GGT GAC GGT GAT
▸Gly Ala Thr Gly Asp Phe Ala Gly Ser Asn Ser Gln Met Ala Gln Val Gly Asp Gly Asp

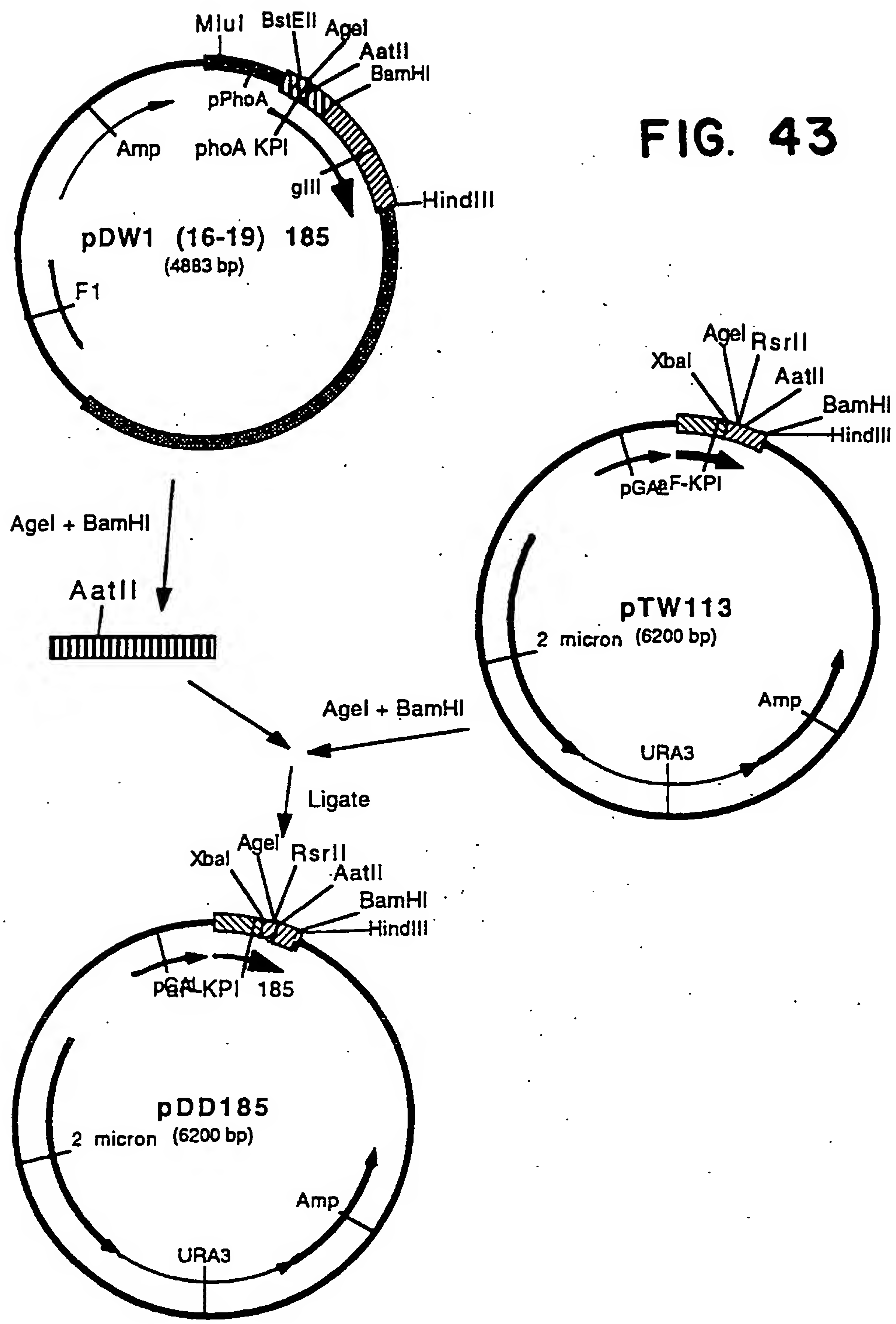
AAT TCA CCT TTA ATG AAT AAT TTC CGT CAA TAT TTA CCT TCC CTC CCT CAA TCG GTT GAA
▸Asn Ser Pro Leu Met Asn Asn Phe Arg Gln Tyr Leu Pro Ser Leu Pro Gln Ser Val Glu

TGT CGC CCT TTT GTC TTT GGC GCT GGT AAA CCA TAC GAA TTT TCT ATT GAT TGT GAC AAA
▸Cys Arg Pro Phe Val Phe Gly Ala Gly Lys Pro Tyr Glu Phe Ser Ile Asp Cys Asp Lys

ATA AAC TTA TTC CGT GGT GTC TTT GCG TTT CTT TTA TAT GTT GCC ACC TTT ATG TAT GTA
▸Ile Asn Leu Phe Arg Gly Val Phe Ala Phe Leu Leu Tyr Val Ala Thr Phe Met Tyr Val

TTT TCT ACG TTT GCT AAC ATA CTG CGT AAT AAG GAG TCT TAA TA
▸Phe Ser Thr Phe Ala Asn Ile Leu Arg Asn Lys Glu Ser

FIG. 43


MluI
BstEII
AgeI
AatII
BamHI
pPhoA
Amp
phoA KPI
gIII
HindIII
pDW1 (16-19) 185
(4883 bp)
F1
AgeI + BamHI
AatII
AgeI + BamHI
Ligate
XbaI
AgeI
RsrII
AatII
BamHI
HindIII
pGAL
αF-KPI
pTW113
2 micron
(6200 bp)
Amp
URA3
XbaI
AgeI
RsrII
AatII
BamHI
HindIII
pGAL
KPI
185
pDD185
2 micron
(6200 bp)
Amp
URA3

pDD185

FIG. 44

```
α-factor
ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▸Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▸Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▸Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▸Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                                    KPI(-4-57; M15A, S17F)
             XbaI
GAA GAA GGG GTA TCT CTA GAT AAA AGA|GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT|CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▸Glu Glu Gly Val Ser Leu Asp Lys Arg|Glu Val Val Arg Glu Val Cys Ser Glu Gln

       RsrII
       AgeI                                                AatII
GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TTC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG AAG GCG ACC ATG AAA CTG CAG TGA CTT
▸Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Phe Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▸Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                    BamHI       HindIII
ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▸Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

Protease inhibition by KPI (-4-57) variants

K_is (nM)

Variant		Substitution 9	15	16	17	18	37	kallikrein	XIIa	Xa
TW113 KPI (-4-57)								45.00	3718.0	161.0
DD185	KPI (-4-57; M15A, S17F)		A		F			0.39	150.0	196.0
TW6165	KPI (-4-57; M15A, S17W)		A		W			0.65	206.0	nd
TW6166	KPI (-4-57; M15A, S17Y)		A		Y			0.40	73.0	nd
TW6175	KPI (-4-57; M15L, S17F)		L		F			0.50	35.0	56.0
BG028	KPI (-4-57; M15L, S17Y)		L		Y			1.10	93.8	nd
TW6183	KPI (-4-57; I16H, S17F)			H	F			1.20	12440.0	159.0
TW6184	KPI (-4-57; I16H, S17Y)			H	Y			0.91	14000.0	214.0
TW6185	KPI (-4-57; I16H, S17W)			H	W			1.30	388.0	473.0
TW6173	KPI (-4-57; M15A, I16H)		A	H				1.00	1432.0	nd
TW6174	KPI (-4-57; M15L, I16H)		L	H				0.90	2796.0	nd
BG015	KPI (-4-57; M15L, S17Y,R18H)		L		Y	H		6.00	19.4	597.0
BG022	KPI (-4-57; M15A,S17Y,R18H)		A		Y	H		0.64	14.5	nd
BG029	KPI (-4-57; T9V, M15L, S17Y,R18H)	V	L		Y	H		3.20	7.9	nd
BG033	KPI (-4-57; T9V, M15A, S17Y,R18H)	V	A		Y	H		0.75	5.8	nd
DD131	KPI (-4-57; M15L, I16F, S17K)		L	F	K			7.90	1385.0	3.3
DD134	KPI (-4-57; M15L, I16F, S17K, G37Y)		L	F	K		Y	1.10	15640.0	0.6
DD135	KPI (-4-57; M15L, I16F, S17K, G37L)		L	F	K		L	1.30	7473.0	0.9

FIG. 45

FIG. 46(I)

Variant	Sequence	Inhibition Ki (nM) P. kalli	Plasmin	XIIa	Xa
Aprotinin	RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA	20.00	0.23	5000.0	
Aprotinin R15, S42	DFCLEPPYTGPCRARIIRYFYNAKAGLCQTFVYGGCRAKSNNFKSAEDCMRTCGGA	0.91	0.17	3983.0	
KPI (-4-57)	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	45.00	34.00	3718.0	161.0
TW6167	EVVREVCSEQAEPGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	61.00		3641.0	288.0
BG031	EVVREVCSEQAEVGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	34.00			
BG032	EVVREVCSEQAESGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	49.00		731.0	
TW101	EVCSEQAETGPCKAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	2000.00	11.50		
TW6208	EVVREVCSEQAETGPCRGMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			369.0	
TW106	EVCSEQAETGPCRARISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	560.00	3.70		
DD108	EVVREVCSEQAETGPCRAAISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.70	11.20	1600.0	123.0
DD109	EVVREVCSEQAETGPCRAIISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	9.50		1681.0	421.0
DD110	EVVREVCSEQAETGPCRALISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	2.10		624.0	55.0
DD111	EVVREVCSEQAETGPCRASISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	5.60			
DD112	EVVREVCSEQAETGPCRAVISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	6.80		998.0	
TW6179	EVVREVCSEQAETGPCRAGISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	78.00		368.0	
TW6163	EVVREVCSEQAETGPCRAMHSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	4.70	103.58	4532.0	457.0
TW6172	EVVREVCSEQAETGPCRAMASRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	315.00			1463.0
TW6180	EVVREVCSEQAETGPCRAMFSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	70.00		885.0	39.0
TW6181	EVVREVCSEQAETGPCRAMKSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	150.00		1514.0	
BG001	EVVREVCSEQAETGPCRAMLSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	38.00	10.00	489.0	204.0
TW116	EVCSEQAETGPCRAMIIRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	145.00	89.00		806.0
DD102	EVVREVCSEQAETGPCRAMIPRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	16.00		315.0	
DD103	EVVREVCSEQAETGPCRAMIFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	17.00		2128.0	110.0
DD104	EVVREVCSEQAETGPCRAMIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	15.00		237.0	345.0
DD105	EVVREVCSEQAETGPCRAMIWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	18.00		198.0	320.0
TW6168	EVVREVCSEQAETGPCRAMILRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	25.80		3521.0	395.0

FIG. 46(2)

Variant	Sequence	Inhibition Ki (nM) P. kalli	Plasmin	XIIa	Xa
TW6182	EVVREVCSEQAETGPCRAMIHRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	36.00		752.0	
TW6194	EVVREVCSEQAETGPCRAMIERWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	70.83			
TW6210	EVVREVCSEQAETGPCRAMIQRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	54.00		277.0	
CL006	EVVREVCSEQAETGPCRAMISAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	110.20		89600.0	133.0
BG012	EVVREVCSEQAETGPCRAMISTWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			40.0	116.0
TW6209	EVVREVCSEQAETGPCRAMISHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	81.00	45.90	184.0	613.0
TW6211	EVVREVCSEQAETGPCRAMISKWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	184.00		402.0	
DD128	EVVREVCSEQAETGPCRAMISLWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	44.00			37.0
TW6142	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFVYGGCGGNRNNFDTEEYCMAVCGSAI	18.00	18.00	7972.0	225.0
AL301	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFLYGGCGGNRNNFDTEEYCMAVCGSAI	216.00		1557.0	
AL302	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFGYGGCGGNRNNFDTEEYCMAVCGSAI	39.00			316.0
TW6147	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCAGNRNNFDTEEYCMAVCGSAI	35.00		1090.0	179.0
TW6138	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCKGNRNNFDTEEYCMAVCGSAI	18.00		921.0	309.0
TW6154	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCLGNRNNFDTEEYCMAVCGSAI	11.00		915.0	39.0
TW6155	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCMGNRNNFDTEEYCMAVCGSAI	11.00			27.0
TW6140	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCNGNRNNFDTEEYCMAVCGSAI	35.00		475.0	
TW6156	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCPGNRNNFDTEEYCMAVCGSAI				
TW6141	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCQGNRNNFDTEEYCMAVCGSAI	42.00			
TW118	EVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	6.00	24.00	13009.0	68.0
DD100	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCCGNRNNFDTEEYCMAVCGSAI	15.00			
TW6157	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCSGNRNNFDTEEYCMAVCGSAI	40.00		511.0	168.0
TW6158	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCTGNRNNFDTEEYCMAVCGSAI	29.00			
TW6159	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCVGNRNNFDTEEYCMAVCGSAI	17.00			64.0
TW6161	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	7.50	18.00	1507.0	8.7
DD101	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCDGNRNNFDTEEYCMAVCGSAI	64.00		924.0	
TW6151	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCEGNRNNFDTEEYCMAVCGSAI	163.00		1162.0	954.0

FIG. 46(3)

Variant	Sequence	Inhibition Ki (nM) P. kalli	Plasmin	XIIa	Xa
TW6139	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCHGNRNNFDTEEYCMAVCGSAI	19.00	22.80	152.0	78.0
TW6153	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCIGNRNNFDTEEYCMAVCGSAI	11.20	21.30	65.0	36.0
TW122	EVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGANRNNFDTEEYCMAVCGSAI	32.00	27.00		581.0
TW6178	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGRNRNNFDTEEYCMAVCGSAI	16.00		444.0	
TW6148	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGARNNFDTEEYCMAVCGSAI	40.00			
TW124	EVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNSNNFDTEEYCMAVCGSAI	64.00	48.00		
TW6149	EVVREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNANNFDTEEYCMAVCGSAI	54.00			
TW6173	EVVREVCSEQAETGPCRAAHSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.00	7.24	1432.0	
TW6174	EVVREVCSEQAETGPCRALHSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.90	6.89	2796.0	
BG002	EVVREVCSEQAETGPCRALLSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.98	19.00	403.0	60.0
DD129	EVVREVCSEQAETGPCRALFSRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	3.60		1864.0	6.0
DD185	EVVREVCSEQAETGPCRAAIFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.39	8.71	150.0	196.0
TW6165	EVVREVCSEQAETGPCRAAIWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.65	16.40	206.0	
TW6166	EVVREVCSEQAETGPCRAAIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.40	10.10	73.0	
BG028	EVVREVCSEQAETGPCRALIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.10	12.10	93.8	
TW6169	EVVREVCSEQAETGPCRALILRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.20		619.0	111.0
DD113	EVVREVCSEQAETGPCRALIPRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.85	12.80	293.0	74.0
TW6175	EVVREVCSEQAETGPCRALIFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.50	7.46	35.0	56.0
TW6201	EVVREVCSEQAETGPCRAGIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	34.60		419.0	
TW6202	EVVREVCSEQAETGPCRAGIWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	128.50		1237.0	
TW6203	EVVREVCSEQAETGPCRAGIPRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	31.20		5045.0	
TW6204	EVVREVCSEQAETGPCRAAISAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			147.0	87.0
TW6205	EVVREVCSEQAETGPCRALISAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			195.0	29.0
DD114	EVVREVCSEQAETGPCRAAISRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	0.70	7.77	224.0	
TW6190	EVVREVCSEQAETGPCRAAISRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	0.83	52.20	589.0	1396.0
TW6183	EVVREVCSEQAETGPCRAMHFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.20	11.68	12440.0	159.0

FIG. 46(4)

Variant	Sequence	Inhibition Ki (nM) P. kalli	Plasmin	XIIa	Xa
TW6184	EVVREVCSEQAETGPCRAMHYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.91	11.96	14000.0	214.0
TW6185	EVVREVCSEQAETGPCRAMHWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.30	18.60	388.0	473.0
BG003	EVVREVCSEQAETGPCRAMLHRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	36.00		467.0	
TW6186	EVVREVCSEQAETGPCRAMHSRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	0.48	8.86	186.0	11.0
TW6187	EVVREVCSEQAETGPCRAMIFRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	3.80	15.40	92.0	15.0
TW6188	EVVREVCSEQAETGPCRAMIYRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	4.00		419.0	24.0
TW6189	EVVREVCSEQAETGPCRAMIWRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	4.00			34.0
TW6170	EVVREVCSEQAEPGPCRALILRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	2.50			452.0
DD115	EVVREVCSEQAETGPCRGYITRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI			213.0	299.0
DD170	EVVREVCSEQAETGPCRALHNRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.99	18.00	550.0	
TW6176	EVVREVCSEQAETGPCRAAHFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	3.50	118.00	56.0	
TW6177	EVVREVCSEQAETGPCRALHFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	7.20	32.70	245.0	156.0
BG006	EVVREVCSEQAETGPCRAALFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.30	12.10	80.0	
DD130	EVVREVCSEQAETGPCRALFTRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	5.50			9.5
DD131	EVVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	7.90	2.00	1385.0	3.3
DD132	EVVREVCSEQAETGPCRAFFKRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	112.00			16.8
DD120	EVVREVCSEQAETGPCRAAFSAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	8.30			11.0
DD121	EVVREVCSEQAETGPCRALLSAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	19.00			21.0
BG014	EVVREVCSEQAETGPCRALIWHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	9.20	18.70	18.0	
DD122	EVVREVCSEQAETGPCRALIFAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	15.00			46.0
BG015	EVVREVCSEQAETGPCRALIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	6.00	12.20	19.4	597.0
BG020	EVVREVCSEQAETGPCRAAIHKWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	1.70		106.0	
BG022	EVVREVCSEQAETGPCRAAIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEHCMAVCGSAI	0.64	7.26	14.5	
BG023	EVVREVCSEQAETGPCRALIQHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	23.00		262.0	
BG024	EVVREVCSEQAETGPCRALIYKWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	4.10	7.47	38.7	
BG027	EVVREVCSEQAETGPCRAAIQHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	5.80		144.0	

FIG. 46(5)

Variant	Sequence	Inhibition Ki (nM) P. kalli	Plasmin	XIIa	Xa
DD116	EVVREVCSEQAETGPCRAAIFRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	0.14		583.0	84.0
TW6191	EVVREVCSEQAETGPCRAAIFRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	0.26		664.0	20.0
DD117	EVVREVCSEQAETGPCRALIPRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	0.11		1034.0	99.0
BG029	EVVREVCSEQAEVGPCRALIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	3.20		7.9	
BG030	EVVREVCSEQAESGPCRALIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	4.60		26.1	
BG033	EVVREVCSEQAEVGPCRAAIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEHCMAVCGSAI	0.75		5.8	
BG034	EVVREVCSEQAESGPCRAAIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	0.47		18.5	
BG040	EVVREVCSEQAEIGPCRALIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	3.40		8.6	
BG016	EVVREVCSEQAETGPCRGAIQHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	160.00		178.0	
BG017	EVVREVCSEQAETGPCRGAIRHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	180.00		200.0	
BG021	EVVREVCSEQAETGPCRGSIRHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	340.00		224.0	
BG025	EVVREVCSEQAETGPCRGLIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	65.00		16.2	
BG026	EVVREVCSEQAETGPCRGAIYHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSAI	50.00		34.9	
DD118	EVVREVCSEQAETGPCRALHNRWYFDVTEGKCAPFFYGGCRGNRNNFDTEEYCMAVCGSAI	0.53			
DD134	EVVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCYGNRNNFDTEEYCMAVCGSAI	1.10	1.05	15640.0	0.6
DD135	EVVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCLGNRNNFDTEEYCMAVCGSAI	1.30		7473.0	0.9
DD136	EVVREVCSEQAETGPCRALFKRWYFDVTEGKCAPFFYGGCMGNRNNFDTEEYCMAVCGSAI	1.10			1.8

FIG. 47

VOLUMES

NS	344.25
KPI	245.75

	KPI	NS
	298	366
	266	342
	354	294
	258	385
	168	288
	266	469
	172	338
	184	272
MEAN	245.75	344.25
STDEV	66.2414415	63.97488346
TTEST		0.009094999

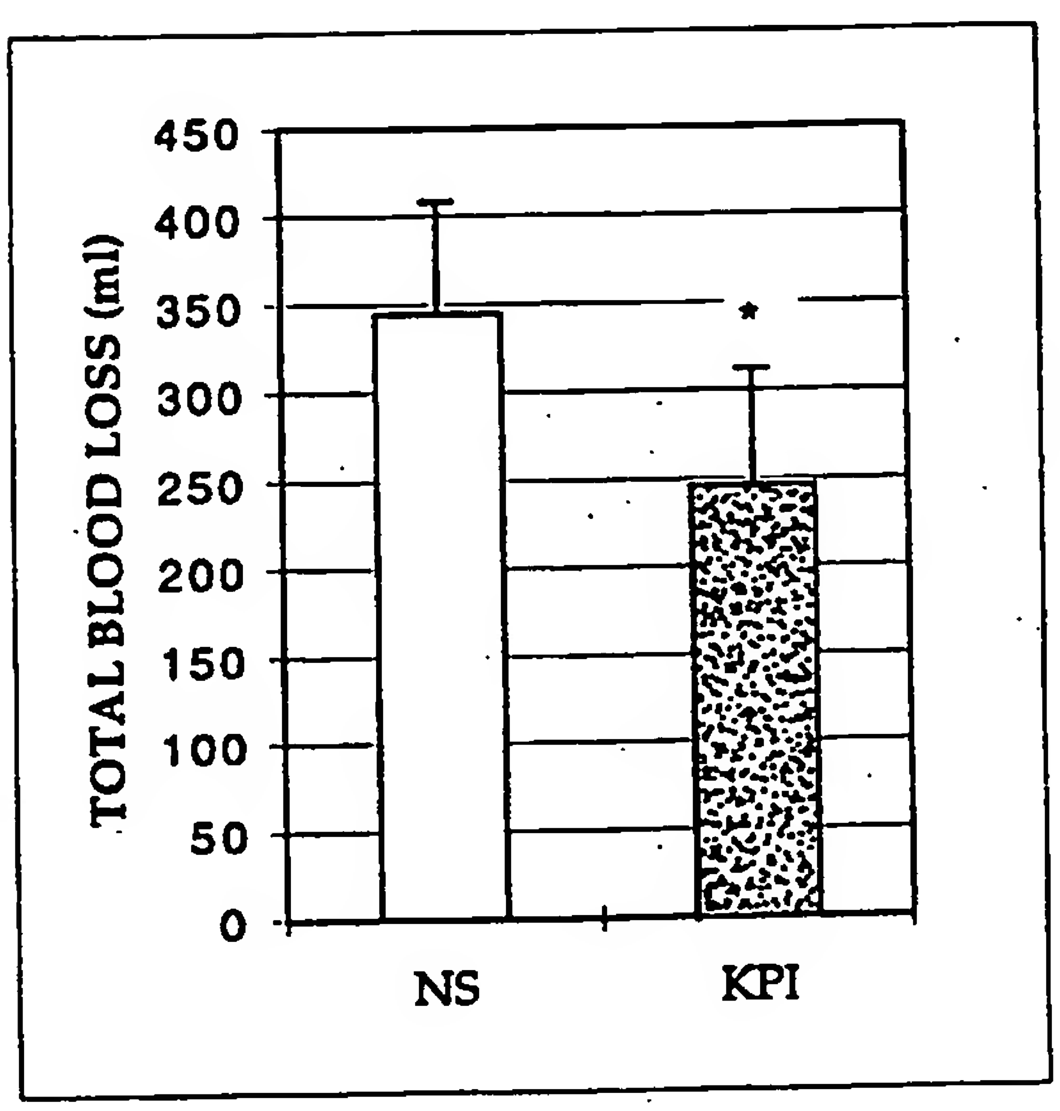


FIG. 48

HEMOGLOBIN

NS	23.61
KPI	13.59

	KPI	NS
	16.58	24.95
	15.19	24.87
	20.21	20.46
	8.99	27.59
	14.63	18.23
	15.31	31.59
	7.7	23.26
	10.14	17.96
MEAN	13.59375	23.61375
STDEV	4.261438	4.68761
TTEST		0.000536

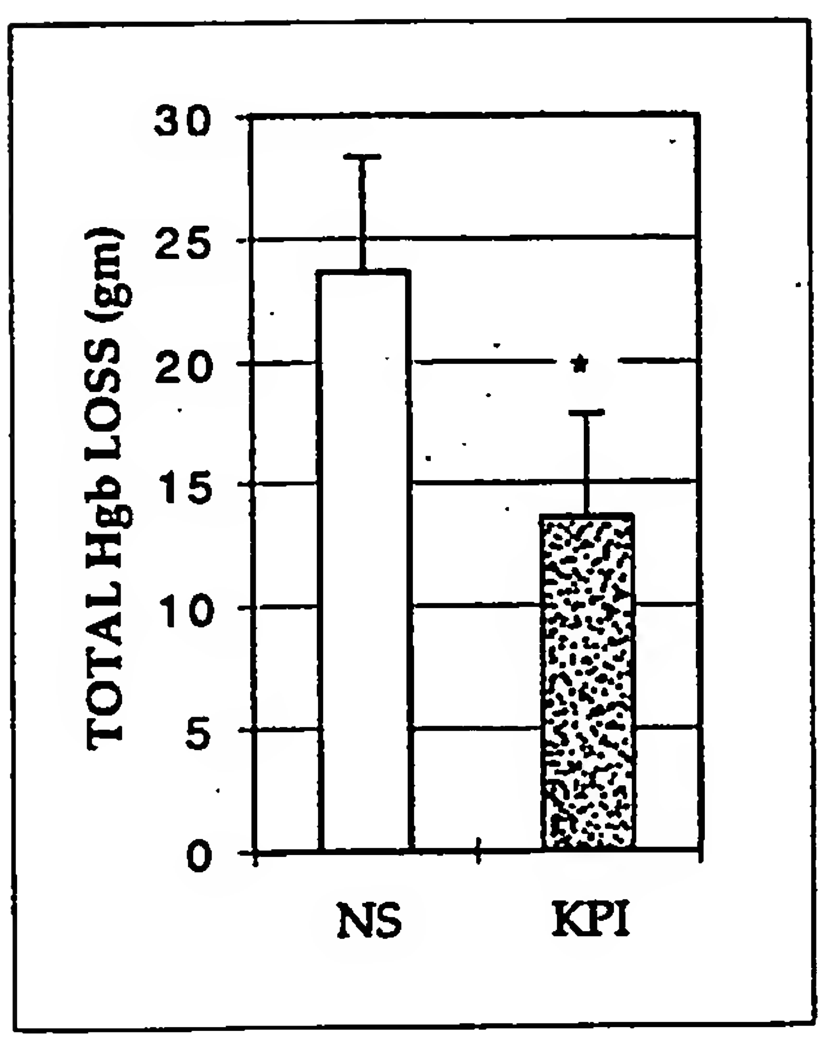


FIG. 49

PaO2

	Baseline PaO2		End CPB		Obs 60 min		Obs 180 min	
	KPI	NS	KPI	NS	KPI	NS	KPI	NS
	652.2	670.9	495.7	60.5	483.7	441.3		391.3
	654	559.2	444.6	132.2	330.1	448.7	264.1	484.6
	596.2	622.9	170.2	93.8	415.4	85.1	416.5	81.3
	606.2	689.2	264.2	333.9	430.2	529.6	361.9	333.2
	633.1	665.1	567.2	341.7	613	568.3	90.8	546.6
	646.6	527	507.4	226.9	564.3	438.1	518.2	485.3
	563.2	461.7	547.1	89.1	501	42.6	494.2	45.6
	659.9	508	416.6	59.7	504.5	405.8	452	383.7
MEAN	626.425	588	426.625	167.225	480.275	369.938	371.1	344
STDEV	34.46923	85.50556	140.4741	117.9931	88.61879	196.5235	150.2774	186.227
TTEST	*p=*	*0.268*	*p=*	*0.0014*	*p=*	0.17915	*p=*	0.76
	N.S.				N.S.			

FIG. 50

Summary of Data

	Total volume loss	Total Hgb Loss	Total Volumes: Chest tube	Total Volumes: Sacrifice	Serial Chest tube Hbg: 0-30min	Serial Chest tube Hbg: 30-60min	Serial Chest tube Hbg: 60-120min	Serial Chest tube Hbg: 120-180min
KPI-1	298	16.58	185	113	3.7	4.3	8.6	6.2
KPI-2	266	15.19	198	68	4.3	6.4	6.7	5.7
KPI-3	354	20.21	142	212	4.1	4.4	7	7.1
KPI-4	258	8.99	190	68	2.8	4	4.4	1.9
KPI-5	168	14.63	96	72	6.3	6.5	7	6.7
KPI-6	266	15.31	188	78	4.1	6.1	5.6	6.3
KPI-7	172	7.7	134	38	3.1	4.6	5.4	4.4
KPI-8	184	10.14	158	26	6.9	5.8	5.4	4.2
MEAN	245.75	13.59			4.41	5.26	6.26	5.3
STDEV	66.24	4.26			1.45	1.04	1.32	1.72
NS-1A	366	24.95	274	92	7.7	8.6	6.1	5.4
NS-2	342	24.87	236	106	7.2	7.4	7.6	7.1
NS-3	294	20.46	252	42	5.4	7.5	7.5	6.5
NS-4	385	27.59	303	82	8.4	7.2	7.1	6.3
NS-5	288	18.23	140	148	7.5	7.2	5.2	5.6
NS-6	469	31.59	261	208	4	7	7.3	7.4
NS-7	338	23.26	218	120	7.5	7.7	5.8	4.2
NS-8	272	17.96	206	66	7.4	8.2	6	5.3
MEAN	344.25	23.61			6.89	7.6	6.58	6.1
STDEV	63.97	4.69			1.44	1.04	0.91	0.85
	*p = 0.009	*p = 0.0005			*p = 0.004	*p = 0002	NS	NS

pTW 6166

FIG. 51

```
 α-factor
 ─────────►
 ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
 TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▸Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

 GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
 CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▸Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

 ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
 TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▸Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

 AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
 TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▸Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

                                      KPI(-4-57; M15A, S17Y)
                         XbaI         ─────────────────────────►
 GAA GAA GGG GTA TCT CTA GAT AAA AGA | GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
 CTT CTT CCC CAT AGA GAT CTA TTT TCT | CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▸Glu Glu Gly Val Ser Leu Asp Lys Arg | Glu Val Val Arg Glu Val Cys Ser Glu Gln

            RsrII
           AgeI                                                  AatII
 GCT GAG ACC GGT CCG TGC CGT GCA GCT ATC TAC CGC TGG TAC TTT GAC GTC ACT GAA
 CGA CTC TGG CCA GGC ACG GCA CGT CGA TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
▸Ala Glu Thr Gly Pro Cys Arg Ala Ala Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu

 GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
 CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▸Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

                                         BamHI           HindIII
 ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
 TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▸Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile
```

FIG. 52

α-factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI — KPI(-4-57; M15L, S17F)

GAA GAA GGG GTA TCT CTA GAT AAA AGA | GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT | CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
Glu Glu Gly Val Ser Leu Asp Lys Arg | Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII
AgeI — AatII

GCT GAG ACC GGT CCG TGC CGT GCA TTG ATC TTC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT AAC TAG AAG GCG ACC ATG AAA CTG CAG TGA CTT
Ala Glu Thr Gly Pro Cys Arg Ala Leu Ile Phe Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI — HindIII

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

FIG. 53

α-factor

ATG AGA TTT CCT TCA ATT TTT ACT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT
TAC TCT AAA GGA AGT TAA AAA TGA CGT CAA AAT AAG CGT CGT AGG AGG CGT AAT CGA
▸Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser Ala Leu Ala

GCT CCA GTC AAC ACT ACA ACA GAA GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC
CGA GGT CAG TTG TGA TGT TGT CTT CTA CTT TGC CGT GTT TAA GGC CGA CTT CGA CAG
▸Ala Pro Val Asn Thr Thr Thr Glu Asp Glu Thr Ala Gln Ile Pro Ala Glu Ala Val

ATC GGT TAC TTA GAT TTA GAA GGG GAT TTC GAT GTT GCT GTT TTG CCA TTT TCC AAC
TAG CCA ATG AAT CTA AAT CTT CCC CTA AAG CTA CAA CGA CAA AAC GGT AAA AGG TTG
▸Ile Gly Tyr Leu Asp Leu Glu Gly Asp Phe Asp Val Ala Val Leu Pro Phe Ser Asn

AGC ACA AAT AAC GGG TTA TTG TTT ATA AAT ACT ACT ATT GCC AGC ATT GCT GCT AAA
TCG TGT TTA TTG CCC AAT AAC AAA TAT TTA TGA TGA TAA CGG TCG TAA CGA CGA TTT
▸Ser Thr Asn Asn Gly Leu Leu Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys

XbaI KPI(-4-57; M15L, S17Y)

GAA GAA GGG GTA TCT CTA GAT AAA AGA GAG GTT GTT AGA GAG GTG TGC TCT GAA CAA
CTT CTT CCC CAT AGA GAT CTA TTT TCT CTC CAA CAA TCT CTC CAC ACG AGA CTT GTT
▸Glu Glu Gly Val Ser Leu Asp Lys Arg Glu Val Val Arg Glu Val Cys Ser Glu Gln

RsrII
AgeI AatII

GCT GAG ACC GGT CCG TGC CGT GCA TTG ATC TAC CGC TGG TAC TTT GAC GTC ACT GAA
CGA CTC TGG CCA GGC ACG GCA CGT AAC TAG ATG GCG ACC ATG AAA CTG CAG TGA CTT
▸Ala Glu Thr Gly Pro Cys Arg Ala Leu Ile Tyr Arg Trp Tyr Phe Asp Val Thr Glu

GGT AAG TGC GCT CCA TTC TTT TAC GGC GGT TGC GGC GGC AAC CGT AAC AAC TTT GAC
CCA TTC ACG CGA GGT AAG AAA ATG CCG CCA ACG CCG CCG TTG GCA TTG TTG AAA CTG
▸Gly Lys Cys Ala Pro Phe Phe Tyr Gly Gly Cys Gly Gly Asn Arg Asn Asn Phe Asp

BamHI HindIII

ACT GAA GAG TAC TGC ATG GCA GTG TGC GGA TCC GCT ATT TAA GCT T
TGA CTT CTC ATG ACG TAC CGT CAC ACG CCT AGG CGA TAA ATT CGA A
▸Thr Glu Glu Tyr Cys Met Ala Val Cys Gly Ser Ala Ile

FIG. 54(I)

PROTEIN	SEQUENCE	K_i kallikrein	K_i Factor XIIa	K_i Plasmin
Aprotinin	RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCHRTCGGA	22.6	5000	0.33
KPI(-4→57)	EVVREVCSEQAETGPCRAHISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	45.0	3718.0	34.00
TW101	EVVREVCSEQAETGPCKAHISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	>5000	nd	12.30
TW106	EVVREVCSEQAETGPCRARISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	449.0	nd	2.98
TW116	EVVREVCSEQAETGPCRAHIIRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	116.00	nd	70.90
TW105	EVVREVCSEQAETGPCKARISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	>5000	nd	1.45
TW117	EVVREVCSEQAETGPCKAHIIRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	>5000	nd	19.90
TW115	EVVREVCSEQAETGPCRARIIRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	671.0	nd	2.24
TW102	EVVREVCSEQAETGPCKARIIRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	>5000	nd	1.27
CL005	EVVREVCSEQAETGPCAAHISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	>5000	>5000	>5000
TW6172	EVVREVCSEQAETGPCRAHASRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	315.0	nd	1555.0
TW6207	EVVREVCSEQAETGPCRAHIARWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	54.0	635.0	44.10
CL0062	EVVREVCSEQAETGPCRAHISAWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	110.2	89600	31.10
DD108	EVVREVCSEQAETGPCRAAISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	1.7	1600.0	11.20
DD110	EVVREVCSEQAETGPCRALISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	2.1	624.0	11.000
DD111	EVVREVCSEQAETGPCRASISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	5.6	nd	nd
DD112	EVVREVCSEQAETGPCRAVISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	6.8	998.0	nd
DD102	EVVREVCSEQAETGPCRAHIPRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	16.0	315.0	nd
DD103	EVVREVCSEQAETGPCRAHIFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	17.0	2128.0	nd
DD104	EVVREVCSEQAETGPCRAHIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	15.0	237.0	nd
DD105	EVVREVCSEQAETGPCRAHIWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	18.0	198.0	nd
TW6166	EVVREVCSEQAETGPCRAAIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	0.4	73.0	10.10

FIG. 54(2)

TW6165	EVVREVCSEQAETGPCRAAIWRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	.65	206.0	16.4
BG028	EVVREVCSEQAETGPCRALIYRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	1.1	93.8	12.10
TW6175	EVVREVCSEQAETGPCRALIFRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	0.5	35.0	7.46
TW6238	EVVREVCSEQAETGPCRAAIWHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	2.5	40.0	nd
TW6245	EVVREVCSEQAETGPCRAAIHQWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	9.9	76	nd
TW6247	EVVREVCSEQAETGPCRAAIWHWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCHAVCGSAI	4.6	38	nd

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(54) Title: KUNITZ TYPE PROTEASE INHIBITORS

(57) Abstract

Analogues of the Kunitz Protease Inhibitor (KPI) domain of amyloid precursor protein bind to and inhibit activity of serine proteases, including kallikrein, plasmin and coagulation factors such as factors VIIa, IXa, Xa, XIa and XIIa. Pharmaceutical compositions containing the KPI analogues, along with methods for using such compositions, are useful for ameliorating and treating clinical conditions associated with increased serine protease activity, such as blood loss related to cardiopulmonary bypass surgery. Nucleic acid sequences encoding these analogues and systems for expression of the peptides of the invention are provided.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/06384

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/15 C07K14/81 C12N1/21 A61K38/57 C12N1/19
C12N15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHEMISTRY 29 (43). 1990. 10018-10022. CODEN: BICHAW ISSN: 0006-2960, XP002015891 HYNES T R ET AL: "X-RAY CRYSTAL STRUCTURE OF THE PROTEASE INHIBITOR DOMAIN OF ALZHEIMER 'S AMYLOID BETA-PROTEIN PRECURSOR." see the whole document ---	1-133
Y	JOURNAL OF MOLECULAR BIOLOGY, vol. 230, 1993, pages 919-933, XP000608106 J. PERONA ET AL: "Crystal structures of rat anionic trypsin complexed with the protein inhibitors APPI and BPTI" see the whole document ---	1-133

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
28 October 1996	04. 11. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van der Schaal, C

INTERNATIONAL SEARCH REPORT

Intern[illegible]nal Application No
PCT/US 96/06384

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BIOLOGICAL CHEMISTRY 269 (35). 1994. 22137-22144. ISSN: 0021-9258, XP002015892 DENNIS M S ET AL: "Kunitz domain inhibitors of tissue factor-factor VIIa: II. Potent and specific inhibitors by competitive phage selection." cited in the application	1,2,68
Y	see table II especially page 22137, right column, fourth paragraph	1-133
X	JOURNAL OF BIOLOGICAL CHEMISTRY 269 (35). 1994. 22129-22136. ISSN: 0021-9258, XP002015893 DENNIS M S ET AL: "Kunitz domain inhibitors of tissue factor-factor VIIa: I. Potent inhibitors selected from libraries by phage display." cited in the application	1,2,68
Y	see table I	1-133
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 31, 5 November 1991, MD US, pages 21011-21013, XP002015894 S. SINHA ET AL: "Conversion of the Alzheimer's beta-Amyloid precursor protein (APP) Kunitz domain into a potent human neutrophil elastase inhibitor" see the whole document	1-133
X	EP,A,0 393 431 (BAYER AG) 24 October 1990	1-3,68, 80
Y	see the whole document	1-133
Y	WO,A,93 14119 (NOVONORDISK AS) 22 July 1993 see the whole document	1-133
Y	WO,A,93 14120 (NOVONORDISK AS) 22 July 1993 see the whole document	1-133
A	BIOCHEM BIOPHYS RES COMMUN 167 (2). 1990. 716-721. CODEN: BBRCA9 ISSN: 0006-291X, XP002015895 KIDO H ET AL: "PROTEASE-SPECIFICITY OF KUNITZ INHIBITOR DOMAIN OF ALZHEIMER 'S DISEASE AMYLOID PROTEIN PRECURSOR." see abstract	

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/06384

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY 270 (43). 1995. 25411-25417. ISSN: 0021-9258, XP002015896 DENNIS M S ET AL: "Potent and Selective Kunitz Domain Inhibitors of Plasma Kallikrein Designed by Phage Display."	1
Y	see table I	1-133

P,X	WO,A,95 23860 (GENENTECH INC) 8 September 1995	1-3,68, 80
Y	see claims	1-133

3

INTERNATIONAL SEARCH REPORT	International application No. PCT/US 96/06384

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 64-67,122-125
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 64-67 and 122-125 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 96/06384

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0393431	24-10-90	DE-A-	3912638	31-10-90
		JP-A-	3041095	21-02-91
WO-A-9314119	22-07-93	AU-B-	671611	05-09-96
		AU-A-	3345793	03-08-93
		CA-A-	2127248	22-07-93
		CZ-A-	9401646	15-12-94
		EP-A-	0621869	02-11-94
		FI-A-	943231	06-07-94
		NO-A-	942552	07-09-94
		NZ-A-	246567	28-05-96
		ZA-A-	9300098	10-08-93
WO-A-9314120	22-07-93	AU-A-	3345893	03-08-93
		CA-A-	2127250	22-07-93
		CZ-A-	9401645	15-12-94
		EP-A-	0621870	02-11-94
		FI-A-	943232	06-07-94
		NO-A-	942551	07-09-94
		NZ-A-	246568	27-02-96
		ZA-A-	9300097	10-08-93
WO-A-9523860	08-09-95	NONE		

B2

CORRECTED VERSION*

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/15, C07K 14/81, A61K 38/57	A3	(11) International Publication Number: WO 97/33996 (43) International Publication Date: 18 September 1997 (18.09.97)

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08/725,251	4 October 1996 (04.10.96)	US

(60) Parent Applications or Grants
(63) Related by Continuation

US	08/725,251 (CIP)
Filed on	4 October 1996 (04.10.96)
US	60/019,793 (CIP)
Filed on	14 June 1996 (14.06.96)
US	60/013,106 (CIP)
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(74) Agents: CHAO, Mark et al.; McDonnell Boehnen Hulbert & Berghoff, 300 South Wacker Drive, Chicago, IL 60606 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

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(88) Date of publication of the international search report: 13 November 1997 (13.11.97)

(54) Title: HUMAN BIKUNIN

(57) Abstract

The instant invention provides for proteins, polypeptides, nucleic acid sequences, constructs, expression vectors, host cells, pharmaceutical compositions of, and methods for using human placental bikunin, serine protease inhibitor domains, and fragments thereof.

*(Referred to in PCT Gazette No. 20/1998, Section II)

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Human Bikunin

Field of the Invention

The compositions of the invention relate to the field of proteins which inhibit serine protease activity. The invention also relates to the field of nucleic acid constructs, vectors and host cells for producing serine protease inhibiting proteins, pharmaceutical compositions containing the protein, and methods for their use.

Background of the Invention

Problem Addressed

Blood loss is a serious complication of major surgeries such as open heart surgery and other complicated procedures. Cardiac surgery patients account for a significant proportion of transfused donor blood. Blood transfusion carries risks of disease transmission and adverse reactions. In addition, donor blood is expensive and demands often exceed supply. Pharmacological methods for reducing blood loss and the resultant need for transfusion have been described (reviewed by Scott et al., Ann. Thorac. Surg. 50: 843-851, 1990).

Protein Serine Protease Inhibitor

Aprotinin, a bovine serine protease inhibitor of the Kunitz family is the active substance in the medicament Trasylol®. Aprotinin (Trasylol®) has been reported as being effective in reducing perioperative blood loss (Royston et al., Lancet ii: 1289-1291, 1987; Dietrich et al., Thorac. Cardiovasc. Surg. 37: 92-98, 1989; Fraedrich et al., Thorac. Cardiovasc. Surg. 37: 89-91, 1989); W. van Oeveren et al. (1987), Ann Thorac. Surg. 44, pp 640-645; Bistrup et al., (1988) Lancet I, 366-367), but adverse effects, including hypotension and flushing (Bohrer et al., Anesthesia 45: 853-854, 1990) and allergic reactions (Dietrich et al., Supra) have been reported. Use of aprotinin in patients previously exposed to it is not recommended (Dietrich et al., Supra). Trasylol® has also been used for the treatment of hyperfibrinolytic hemorrhages and traumatic hemorrhagic shock.

Aprotinin is known to inhibit several serine proteases including trypsin, chymotrypsin, plasmin and kallikrein, and is used therapeutically in the treatment of acute pancreatitis, various states of shock syndrome, hyperfibrinolytic hemorrhage and myocardial infarction (Trapnell et al., (1974) Brit J. Surg. 61: 177; J. McMichan et al., (1982) Circulatory Shock 9: 107; Auer et

al., (1979) Acta Neurochir. 49: 207; Sher (1977) Am J. Obstet. Gynecol. 129: 164; Schneider (1976) , Artzneim.-Firsch. 26: 1606). It is generally thought that Trasylol® reduces blood loss *in vivo* through inhibition of kallikrein and plasmin. It has been found that aprotinin (3-58, Arg15, Ala17, Ser42) exhibits improved plasma kallikrein inhibitory potency as compared to native aprotinin itself (WO 89/10374).

Problems With Aprotinin

Because aprotinin is of bovine origin, there is a finite risk of inducing anaphylaxis in human patients upon re-exposure to the drug. Thus, a human functional equivalent to aprotinin, by virtue of a lower risk of anaphylaxis, would be most useful and desirable to have.

Aprotinin is also nephrotoxic in rodents and dogs when administered repeatedly at high dose (Bayer, Trasylol®, Inhibitor of proteinase; Glasser et al., in "Verhandlungen der Deutschen Gesellschaft fur Innere Medizin, 78. Kongress", Bergmann, Munchen, 1972 pp. 1612-1614). One hypothesis ascribes this effect to the accumulation of aprotinin in the negatively charged proximal tubules of the kidney, due to its high net positive charge (WO 93/14120).

Accordingly, an object of the present invention is to identify human proteins with functional activity similar to aprotinin. It was also an object of the instant invention to identify human proteins, that would be less charged, yet exhibit the same, highly similar, or improved protease specificities as found for aprotinin, especially with respect to the potency of plasmin and kallikrein inhibition. Such inhibitors could then be used repeatedly as medicaments in human patients with reduced risk of adverse immune response and reduced nephrotoxicity.

Brief Summary of the Invention

The instant invention provides for a purified human serine protease inhibitor which can specifically inhibit kallikrein, that has been isolated from human placental tissue via affinity chromatography.

The instant invention provides a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. In one particular embodiment, the instant invention embodies a protein having the amino acid sequence:

```
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS                        179
```
(SEQ ID NO: 1)

In a prefered embodiment the instant invention provides for native human placental bikunin protein having the amino acid sequence:

```
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK                                  170
```
(SEQ ID NO: 52)

In one aspect, the biological activity of the protein of the instant invention is that it can bind to and substantially inhibit the biological activity of trypsin, human plasma and tissue kallikreins, human plasmin and Factor XIIa. In a preferred embodiment, the present invention provides for a native human placental bikunin protein, in glycosylated form. In a further embodiment the instant invention encompasses native human bikunin protein which has been formed such that it contains at least one cysteine-cysteine disulfide bond. In a preferred embodiment, the protein contains at least one intra-chain cysteine-cysteine disulfide bond formed between a pair of cysteines selected from the group consisting of CYS11-CYS61, CYS20-CYS44, CYS36-CYS57, CYS106-CYS156, CYS115-CYS139, and CYS131-CYS152, wherein the cysteines are numbered according to the amino acid sequence of native human placental bikunin. One of ordinary skill will recognize that the protein of the instant invention may fold into the proper three-dimensional conformation, such that the biological activity of native human bikunin is maintained, where none, one or more, or all of the native intra-chain cysteine-cysteine disulfide bonds are present. In a most preferred embodiment, the protein of the instant invention is properly folded and is formed with all of the proper native cysteine-cysteine disulfide bonds.

Active protein of the instant invention can be obtained by purification from human tissue, such as placenta, or via synthetic protein chemistry techniques, as illustrated by the Examples below. It is also understood that the

protein of the instant invention may be obtained using molecular biology techniques, where self-replicating vectors are capable of expressing the protein of the instant invention from transformed cells. Such protein can be made as non-secreted, or secreted forms from transformed cells. In order to facilitate secretion from transformed cells, to enhance the functional stability of the translated protein, or to aid folding of the bikunin protein, certain signal peptide sequences may be added to the NH2-terminal portion of the native human bikunin protein.

In one embodiment, the instant invention thus provides for the native human bikunin protein with at least a portion of the native signal peptide sequence intact. Thus one embodiment of the invention provides for native human bikunin with at least part of the signal peptide, having the amino acid sequence:

```
AGSFLAWLGSLLLSGVLA                                   -1
ADRERSIHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN   50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF  100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE  150
ACMLRCFRQQENPPLPLGSKVVVLAGAVS                       179
```

(SEQ ID NO: 2)

In a prefered embodiment the instant invention provides for a native human placental bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with an intact leader segment having the amino acid sequence:

MAQLCGL RRSRAFLALL GSLLLSGVLA -1

(SEQ ID NO: 53)

In another embodiment, the instant invention provides for bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with the intact leader segment having the amino acid sequence:

MLR AEADGVSRLL GSLLLSGVLA -1

(SEQ ID NO: 54)

In a preferred numbering system used herein the amino acid numbered +1 is assigned to the NH2-terminus of the amino acid sequence for native

human placental bikunin. One will readily recognize that functional protein fragments can be derived from native human placental bikunin, which will maintain at least part of the biological activity of native human placental bikunin, and act as serine protease inhibitors.

In one embodiment, the protein of the instant invention comprises a fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 7-159, hereinafter called "bikunin (7-159)". Thus the instant invention embodies a protein having the amino acid sequence:

```
IHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN           50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF    100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE    150
ACMLRCFRQ                                             159
```

(SEQ ID NO: 3)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another functional variant of this embodiment can be the fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 11-156, bikunin (11-156)

```
CLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN               50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF    100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE    150
ACMLRC                                                156
```

(SEQ ID NO: 50).

One can recognize that the individual Kunitz-like domains are also fragments of the native placental bikunin. In particular, the instant invention provides for a protein having the amino acid sequence of a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 7-64, hereinafter called "bikunin (7-64)". Thus in one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

IHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKCATV 64
(SEQ ID NO: 4)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of the protein of the instant invention can be a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 11-61, "bikunin (11-61)" having the amino acid sequence:

CLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKC 61
(SEQ ID NO: 5)

The instant invention also provides for a protein having the amino acid sequence of a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 102-159, hereinafter called "bikunin (102-159)". Thus one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

YEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRCFRQ 159
(SEQ ID NO: 6)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of this domain can be a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 106-156, "bikunin (106-156)" having the amino acid sequence:

CTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRC 156
(SEQIDNO:7)

Thus one of ordinary skill will recognize that fragments of the native

human bikunin protein can be made which will retain at least some of the native protein biological activity. Such fragments can also be combined in different orientations or multiple combinations to provide for alternative proteins which retain some of, the same, or more biological activity of the native human bikunin protein.

One will readily recognize that biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional Kunitz-like domains from other sources. Biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional protein domains from other sources with a variety of biological activities. The biological activity of the protein of the instant invention can be combined with that of other known protein or proteins to provide for multifunctional fusion proteins having predictable biological activity. Thus, in one embodiment, the instant invention encompasses a protein which contains at least one amino acid sequence segment the same as, or functionally equivalent to the amino acid sequence of either SEQ ID NO: 5 or SEQ ID NO: 7.

An open reading frame which terminates at an early stop codon can still code for a functional protein. The instant invention encompasses such alternative termination, and in one embodiment provides for a protein of the amino acid sequence:

```
ADRERSIHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN     50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDS             92
```
(SEQIDNO:8)

In one embodiment, the instant invention provides for substantially purified, or recombinantly produced native human bikunin protein with an intact segment of the leader sequence, and at least a portion of the native transmembrane region intact. Thus one embodiment of the invention provides for native human bikunin, with an intact leader sequence, and with at least part of the transmembrane domain (underlined), having an amino acid sequence selected from:

```
1)EST                              MLR AEADGVSRLL GSLLLSGVLA    -1
2)PCR                          MAQLCGL RRSRAFLALL GSLLLSGVLA    -1
3)λcDNA                        MAQLCGL RRSRAFLALL GSLLLSGVLA    -1

1)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN   50
2)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN   50
3)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN   50

1)YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF  100
2)YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF  100
3)YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF  100

1)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE  150
2)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE  150
3)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE  150

1)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN  200
2)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN  200
3)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN  200

1)QERALRTVWS SGDDKEQLVK NTYVL                             225
2)QERALRTVWS FGD                                          213
3)QERALRTVWS SGDDKEQLVK NTYVL                             225
```

where sequence 1) is EST derived consensus SEQ ID NO: 45, 2) is PCR clone SEQ ID NO:47, and 3) is lambda cDNA clone SEQ ID NO:49. In a preferred embodiment a protein of the instant invention comprises one of the amino acid sequence of SEQ ID NO: 45, 47 or 49 wherein the protein has been cleaved in the region between the end of the last Kunitz domain and the transmembrane region.

The instant invention also embodies the protein wherein the signal peptide is deleted. Thus the instant invention provides for a protein having the amino acid sequence of SEQ ID NO: 52 continuous with a transmembrane amino acid sequence:

```
EST      VVVLAGLFVM VLILFLGASM VYLIRVARRN      200
EST      QERALRTVWS SGDDKEQLVK NTYVL           225
```

(SEQ ID NO: 69)

a transmembrane amino acid sequence:

```
PCR      VVVLAGLFVM VLILFLGASM VYLIRVARRN      200
PCR      QERALRTVWS FGD                        213
```

(SEQ ID NO: 68)

or a transmembrane amino acid sequence:

```
λcDNA    VVVLAGLFVM VLILFLGASM VYLIRVARRN      200
λcDNA    QERALRTVWS SGDDKEQLVK NTYVL           225
```

(SEQ ID NO: 67) .

The protein amino acid sequences of the instant invention clearly teach one of the art the appropriate nucleic acid sequences which can be used in molecular biology techniques to produce the proteins of the instant invention. Thus, one embodiment of the instant invention provides for a nucleic acid sequence which encodes for a human bikunin having the consensus DNA sequence of Figure 3 (SEQ ID NO: 9), which translates into the amino acid sequence for native human placental bikunin sequence of Figure 3 (SEQ ID NO: 10). In another embodiment, the instant invention provides for a consensus nucleic acid sequence of Figure 4C (SEQ ID NO: 51) which encodes for an amino acid sequence of Figure 4D (SEQ ID NO: 45).

In a preferred embodiment, the instant invention provides for a nucleic acid sequence which encodes for native human placental bikunin having the DNA sequence of Figure 4F (SEQ ID NO: 48) which encodes for the protein sequence of SEQ ID NO: 49. In an another embodiment, the instant invention provides for a nucleic acid sequence of Figure 4E (SEQ ID NO: 46) which encodes for a protein sequence of SEQ ID NO: 47.

One can easily recognize that certain allelic mutations, and conservative substitutions made in the nucleic acid sequence can be made which will still result in a protein amino acid sequence encompassed by the instant invention. One of skill in the art can recognize that certain natural allelic mutations of the protein of the instant invention, and conservative substitutions of amino acids in the protein of the instant invention will not significantly alter the biological activity of the protein, and are encompassed by the instant invention.

The instant invention also provides for pharmaceutical compositions containing human placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery.

The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention in a biologically compatible vehicle is administered to the patient.

The present invention also provides for variants of placental bikunin, and the specific Kunitz domains described above, that contain amino acid substitutions that alter the protease specificity. Preferred sites of substitution are indicated below as positions Xaa^{1} through Xaa^{32} in the amino acid sequence for native placental bikunin. Substitutions at Xaa^{1} through Xaa^{16} are also preferred for variants of bikunin (7-64), while substitutions at Xaa^{17} through Xaa^{32} are preferred for variants of bikunin (102-159).

Thus the present invention embodies protein having an amino acid sequence:

```
Ala Asp Arg Glu Arg Ser Ile Xaa1 Asp Phe                        10
Cys Leu Val Ser Lys Val Xaa2 Gly Xaa3 Cys                       20
Xaa4 Xaa5 Xaa6 Xaa7 Xaa8 Xaa9 Trp Trp Tyr Asn                   30
Val Thr Asp Gly Ser Cys Gln Leu Phe Xaa10                       40
Tyr Xaa11 Gly Cys Xaa12 Xaa13 Xaa14 Ser Asn Asn                 50
Tyr Xaa15 Thr Lys Glu Glu Cys Leu Lys Lys                       60
Cys Ala Thr Xaa16 Thr Glu Asn Ala Thr Gly                       70
Asp Leu Ser Thr Ser Arg Asn Ala Ala Asp                         80
Ser Ser Val Pro Ser Ala Pro Arg Arg Gln                         90
Asp Ser Glu His Asp Ser Ser Asp Met Phe                        100
Asn Tyr Xaa17 Glu Tyr Cys Thr Ala Asn Ala                      110
Val Xaa18 Gly Xaa19 Cys Xaa20 Xaa21 Xaa22 Xaa23 Xaa24          120
Xaa25 Trp Tyr Phe Asp Val Glu Arg Asn Ser                      130
Cys Asn Asn Phe Xaa26 Tyr Xaa27 Gly Cys Xaa28                  140
Xaa29 Xaa30 Lys Asn Ser Tyr Xaa31 Ser Glu Glu                  150
Ala Cys Met Leu Arg Cys Phe Arg Xaa32 Gln                      160
Glu Asn Pro Pro Leu Pro Leu Gly Ser Lys                        170
Val Val Val Leu Ala Gly Ala Val Ser                            179
(SEQ ID NO: 11).
```

where Xaa^1 - Xaa^{32} each independently represents a naturally occurring amino acid residue except Cys, with the proviso that at least one of the amino acid residues Xaa^1-Xaa^{32} is different from the corresponding amino acid residue of the native sequence.

In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e., Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor specificity profile of native placental bikunin or that of the individual Kunitz-like domains, bikunin(7-64) or bikunin (102-159) so that it preferentially inhibits other serine proteases such as, but not limited to, the enzymes of the complement cascade, TF/FVIIa, FXa, thrombin, neutrophil elastase, cathepsin G or proteinase-3.

Examples of preferred variants of placental bikunin include those

wherein Xaa^1 is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Val or Lys, in particular wherein Xaa^1 is His or Pro; or wherein Xaa^2 is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala, Lys, in particular wherein Xaa^2 is Val or Thr; or wherein Xaa^3 is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu, Thr, in particular wherein Xaa^3 is Arg or Pro; or wherein Xaa^4 is an amino acid residue selected from the group consisting of Arg, Lys and Ser, Gln, in particular wherein Xaa^4 is Arg or Lys; or wherein Xaa^5 is an amino acid residue selected from the group consisting of Ala, Gly, Asp, Thr, in particular wherein Xaa^5 is Ala; or wherein Xaa^6 is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg, Phe, in particular wherein Xaa^6 is Ser or Arg ; or wherein Xaa^7 is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa^7 is Met or Ile; or wherein Xaa^8 is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Gln, Asn, Leu, Ser or Ile, in particular wherein Xaa^8 is Pro or Ile; or wherein Xaa^9 is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa^9 is Arg: or wherein Xaa^{10} is an amino acid residue selected from the group consisting of Val, Ile, Lys, Ala, Pro, Phe, Trp, Gln, Leu and Thr, in particular wherein Xaa^{10} is Val; or wherein Xaa^{11} is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa^{11} is Gly; or wherein Xaa^{12} is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gln, Gly, in particular wherein Xaa^{12} is Asp or Arg; or wherein Xaa^{13} is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa^{14} is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa^{15} is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa^{15} is Leu or Lys; or wherein Xaa^{16} is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Val, in particular wherein Xaa^{16} is Val or Ala; or wherein Xaa^{17} is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Lys and Val, in particular wherein Xaa^{17} is Glu or Pro; or wherein Xaa^{18} is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala or Lys, in particular wherein Xaa^{18} is Thr; or wherein Xaa^{19} is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu or Thr, in particular wherein Xaa^{19} is Pro; or wherein Xaa^{20} is an amino acid residue selected from the group consisting of Arg, Lys, Gln and

Ser, in particular wherein Xaa^{20} is Arg or Lys; or wherein Xaa^{21} is an amino acid residue selected from the group consisting of Ala, Asp, Thr or Gly ; in particular wherein Xaa^{21} is Ala; or wherein Xaa^{22} is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg or Phe, in particular wherein Xaa^{22} is Ser or Arg ; or wherein Xaa^{23} is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa^{23} is Phe or Ile; or wherein Xaa^{24} is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Asn, Leu, Gln, Ser or Ile, in particular wherein Xaa^{24} is Pro or Ile; or wherein Xaa^{25} is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa^{25} is Arg: or wherein Xaa^{26} is an amino acid residue selected from the group consisting of Val, Ile, Lys, Leu, Ala, Pro, Phe, Gln, Trp and Thr, in particular wherein Xaa^{26} is Val or Ile; or wherein Xaa^{27} is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa^{27} is Gly; or wherein Xaa^{28} is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gly or Gln, in particular wherein Xaa^{28} is Arg; or wherein Xaa^{29} is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa^{30} is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa^{31} is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa^{31} is Arg or Lys; or wherein Xaa^{32} is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Thr, in particular wherein Xaa^{32} is Gln or Ala.

Description of the Drawings

The invention will be better understood from a consideration of the following detailed description and claims, taken in conjunction with the drawings, in which:

Figure 1 depicts the nucleotide sequence of EST R35464 (SEQ ID NO: 12) and the translation of this DNA sequence (SEQ ID NO: 13) which yielded an open reading frame with some sequence similarity to aprotinin. The translation product contains 5 of the 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). The position normally occupied by the remaining cysteine (at codon 38) contained instead a phenylalanine (indicated by an asterisk).

Figure 2 depicts the nucleotide sequence of EST R74593 (SEQ ID NO: 14),

and the translation of this DNA sequence (SEQ ID NO: 15) which yielded an open reading frame with homology to the Kunitz class of serine protease inhibitor domains. The translation product contained 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). However, this reading frame sequence includes stop codons at codon 3 and 23.

Figure 3 depicts a deduced nucleic acid sequence of human placental bikunin (SEQ ID NO: 9) labeled "consensus" and matched with the translated protein amino acid sequence labeled "translated" (SEQ ID NO: 10). Also as comparison are shown the nucleic acid sequence for ESTs H94519 (SEQ ID NO: 16), N39798 (SEQ ID NO: 17), R74593 (SEQ ID NO: 14) and R35464 (SEQ ID NO: 12). The underlined nucleotides in the consensus sequence correspond to the site of PCR primers described in the Examples. Underlined amino acids in the translated consensus sequence are residues whose identity have been confirmed by amino acid sequencing of purified native human placental bikunin. Nucleotide and amino acid code are standard single letter code, "N" in the nucleic acid code indicates an unassigned nucleic acid, and "*" indicates a stop codon in the amino acid sequence.

Figure 4A depicts the original overlay of a series of ESTs with some nucleic acid sequence homology to ESTs encoding human placental bikunin, or portions thereof. Shown for reference are the relative positions of bikunin (7-64) and bikunin (102-159), labeled KID1 and KID2 respectively.

Figure 4B depicts a subsequent more comprehensive EST overlay incorporating additional ESTs. Numbers on the upper X-axis refer to length in base pairs, starting at the first base from the most 5' EST sequence. The length of each bar is in proportion to the length in base pairs of the individual ESTs including gaps. The EST accession numbers are indicated to the right of their respective EST bars.

Figure 4C depicts the corresponding alignment of the oligonucleotide sequences of each of the overlapping ESTs shown schematically in Figure 4B. The upper sequence (SEQ ID NO: 51) labeled bikunin represents the consensus oligonucleotide sequence derived from the overlapping nucleotides at each position. The numbers refer to base-pair position within the EST map. The oligonucleotides in EST R74593 that are bold underlined (at map positions 994 and 1005) are base insertions observed in R74593 that were consistently absent in each of the other overlapping ESTs.

Figure 4D depicts the amino acid translation of the consensus

oligonucleotide sequence for bikunin depicted in Figure 4C (SEQ ID NO: 45).

Figure 4E depicts the nucleotide sequence (SEQ ID NO: 46) and corresponding amino acid translation (SEQ ID NO: 47) of a placental bikunin encoding sequence that was derived from a human placental cDNA library by PCR-based amplification.

Figure 4F depicts the nucleotide sequence (SEQ ID NO: 48) and corresponding amino acid translation (SEQ ID NO: 49) of a native human placental bikunin encoding clone that was isolated from a human placental lambda cDNA library by colony hybridization.

Figure 4G compares the alignment of the amino acid translated oligonucleotide sequences for placental bikunin obtained by EST overlay (SEQ ID NO: 45), PCR based cloning (SEQ ID NO: 47), and conventional lambda colony hybridization (SEQ ID NO: 49).

Figure 5 shows a graph of purification of human placental bikunin from placental tissue after Superdex 75 Gel-Filtration. The plot is an overlay of the protein elution profile as measured by OD 280 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 6 shows a graph which plots the purification of human placental bikunin from placental tissue using C18 Reverse-Phase Chromatography. The plot is an overlay of the protein elution profile as measured by OD 215 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 7 depicts a silver stained SDS-PAGE gel of highly purified placental bikunin (lane 2), and a series of molecular size marker proteins (lane 1) of the indicated sizes in kilodaltons. Migration was from top to bottom.

Figure 8 shows the amount of trypsin inhibitory activity present in the cell-free fermentation broth from the growth of yeast strains SC101 (panel 8A) or WHL341 (panel 8B) that were stably transformed with a plasmid (pS604) that directs the expression of placental bikunin (102-159).

Figure 9 shows both a silver stained SDS-PAGE (left panel) and a Western blot with anti-placental bikunin (102-159) pAb (right panel) of cell-free fermentation broth from the growth of yeast strain SC101 (recombinants 2.4 and 2.5) that was stably transformed with a plasmid directing the expression of either bovine aprotinin, or placental bikunin (102-159). Migration was from top

to bottom.

Figure 10 is a photograph which shows a silver stained SDS-PAGE of highly purified placental bikunin (102-159) (lane 2) and a series of molecular size marker proteins (lane 1) of the indicated sizes in Kilodaltons. Migration was from top to bottom.

Figure 11 is a photograph which shows the results of Northern blots of mRNA from various human tissues that was hybridized to a ^{32}P labeled cDNA probe encoding either placental bikunin (102-159) (panel 11A) or encoding placental bikunin (1-213) (panel 11B). Migration was from top to bottom. The numbers to the right of each blot refer to the size in kilobases of the adjacent RNA markers. The organs from which mRNA was derived is described under each lane of the blot.

Figure 12 depicts an immunoblot of placental derived placental bikunin with rabbit antiserum raised against either synthetic reduced placental bikunin (7-64) (panel A) or 102-159 (panel B). For each panel, contents were: molecular size markers (lanes 1); native placental bikunin isolated from human placenta (lanes 2); synthetic placental bikunin (7-64) (lanes 3) and synthetic placental bikunin (102-159) (lanes 4). Tricine 10-20% SDS-PAGE gels were blotted and developed with protein A-purified primary polyclonal antibody (8 ug IgG in 20 ml 0.1% BSA/Tris-buffered saline (pH 7.5), followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Migration was from top to bottom.

Figure 13 depicts a Coomassie Blue stained 10-20% Tricine SDS-PAGE gel of 3 micrograms of highly purified placental bikunin (1-170) derived from a baculovirus / Sf9 expression system (lane 2). Lane 1 contains molecular size markers. Migration was from top to bottom.

Figure 14 depicts a comparison of the effect of increasing concentrations of either Sf9-derived human placental bikunin (1-170) (filled circles), synthetic placental bikunin (102-159) (open circles), or aprotinin (open squares) on the activated partial thromboplastin time of human plasma. Clotting was initiated with $CaCl_2$. The concentration of proteins are plotted versus the -fold prolongation in clotting time. The uninhibited clotting time was 30.8 seconds.

Detailed Description of the Invention

The present invention encompasses a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. The instant invention also encompasses

pharmaceutical compositions containing placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery, or with major trauma.

The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery or due to major trauma, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention, in a biologically compatible vehicle, is administered to the patient.

A preferred application for placental bikunin, isolated domains, and other variants is for the reduction of blood loss resulting from trauma or surgery that has the potential for loss of large volumes of blood. These methods and compositions reduce or eliminate the need for whole donor blood or blood products, thereby reducing the risk of infection and other adverse side effects , as well as the cost of surgery. The methods are thus useful in reducing blood loss in normal patients, i.e., those not suffering from inborn or other pre-operative deficiencies in coagulation factors. The reduction in blood loss is seen as a reduction in blood loss during surgery , as reduced post surgical drainage or both. Preferred surgical applications include but are not limited to use in thoracic and abdominal surgery, total and partial hip replacement surgeries and surgeries to treat a patient having an epithelial lesion of the eye. Preferred thoracic surgical procedures include but are not limited to aortocoronary bypass, excision of cardiac and aortic aneurysms, and surgery for esophageal varices, and coronary artery bypass surgery. Preferred abdominal surgeries include but are not limited to liver transplants, radical prostatectomy, surgery for diverticulitis of colon, tumor debulking, surgery on the abdominal aorta and surgery for duodenal ulcers, and repair of liver or spleen trauma. Preferred use for the treatment of trauma include but are not limited to the use in stabilization of severely injured patients at accident sites suffering from e.g., limb loss or major thoracic /abdominal wounds. In case of use for the reduction of blood loss resulting from surgery it is preferred to administer the placental bikunin, isolated domains, or other variant prior to and during surgery, whereas in case of use in trauma settings the placental bikunin variant, isolated domain or other variant is to be administered as rapidly as possible following injury, and should be contained on emergency vehicles traveling to the accident sites.

Factor XII (also known as Hageman Factor) is a serine protease that is found in the circulation in a zymogen form (80 kD) at approximately 29-40

CORRECTED VERSION*

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(57) Abstract

The instant invention provides for proteins, polypeptides, nucleic acid sequences, constructs, expression vectors, host cells, pharmaceutical compositions of, and methods for using human placental bikunin, serine protease inhibitor domains, and fragments thereof.

Human Bikunin

Field of the Invention

The compositions of the invention relate to the field of proteins which inhibit serine protease activity. The invention also relates to the field of nucleic acid constructs, vectors and host cells for producing serine protease inhibiting proteins, pharmaceutical compositions containing the protein, and methods for their use.

Background of the Invention

Problem Addressed

Blood loss is a serious complication of major surgeries such as open heart surgery and other complicated procedures. Cardiac surgery patients account for a significant proportion of transfused donor blood. Blood transfusion carries risks of disease transmission and adverse reactions. In addition, donor blood is expensive and demands often exceed supply. Pharmacological methods for reducing blood loss and the resultant need for transfusion have been described (reviewed by Scott et al., Ann. Thorac. Surg. 50: 843-851, 1990).

Protein Serine Protease Inhibitor

Aprotinin, a bovine serine protease inhibitor of the Kunitz family is the active substance in the medicament Trasylol®. Aprotinin (Trasylol®) has been reported as being effective in reducing perioperative blood loss (Royston et al., Lancet ii: 1289-1291, 1987; Dietrich et al., Thorac. Cardiovasc. Surg. 37: 92-98, 1989; Fraedrich et al., Thorac. Cardiovasc. Surg. 37: 89-91, 1989); W. van Oeveren et al. (1987), Ann Thorac. Surg. 44, pp 640-645; Bistrup et al., (1988) Lancet I, 366-367), but adverse effects, including hypotension and flushing (Bohrer et al., Anesthesia 45: 853-854, 1990) and allergic reactions (Dietrich et al., Supra) have been reported. Use of aprotinin in patients previously exposed to it is not recommended (Dietrich et al., Supra). Trasylol® has also been used for the treatment of hyperfibrinolytic hemorrhages and traumatic hemorrhagic shock.

Aprotinin is known to inhibit several serine proteases including trypsin, chymotrypsin, plasmin and kallikrein, and is used therapeutically in the treatment of acute pancreatitis, various states of shock syndrome, hyperfibrinolytic hemorrhage and myocardial infarction (Trapnell et al., (1974) Brit J. Surg. 61: 177; J. McMichan et al., (1982) Circulatory Shock 9: 107; Auer et

al., (1979) Acta Neurochir. 49: 207; Sher (1977) Am J. Obstet. Gynecol. 129: 164; Schneider (1976), Artzneim.-Firsch. 26: 1606). It is generally thought that Trasylol® reduces blood loss *in vivo* through inhibition of kallikrein and plasmin. It has been found that aprotinin (3-58, Arg15, Ala17, Ser42) exhibits improved plasma kallikrein inhibitory potency as compared to native aprotinin itself (WO 89/10374).

Problems With Aprotinin

Because aprotinin is of bovine origin, there is a finite risk of inducing anaphylaxis in human patients upon re-exposure to the drug. Thus, a human functional equivalent to aprotinin, by virtue of a lower risk of anaphylaxis, would be most useful and desirable to have.

Aprotinin is also nephrotoxic in rodents and dogs when administered repeatedly at high dose (Bayer, Trasylol®, Inhibitor of proteinase; Glasser et al., in "Verhandlungen der Deutschen Gesellschaft fur Innere Medizin, 78. Kongress", Bergmann, Munchen, 1972 pp. 1612-1614). One hypothesis ascribes this effect to the accumulation of aprotinin in the negatively charged proximal tubules of the kidney, due to its high net positive charge (WO 93/14120).

Accordingly, an object of the present invention is to identify human proteins with functional activity similar to aprotinin. It was also an object of the instant invention to identify human proteins, that would be less charged, yet exhibit the same, highly similar, or improved protease specificities as found for aprotinin, especially with respect to the potency of plasmin and kallikrein inhibition. Such inhibitors could then be used repeatedly as medicaments in human patients with reduced risk of adverse immune response and reduced nephrotoxicity.

Brief Summary of the Invention

The instant invention provides for a purified human serine protease inhibitor which can specifically inhibit kallikrein, that has been isolated from human placental tissue via affinity chromatography.

The instant invention provides a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. In one particular embodiment, the instant invention embodies a protein having the amino acid sequence:

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS 179
(SEQ ID NO: 1)

In a prefered embodiment the instant invention provides for native human placental bikunin protein having the amino acid sequence:

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK 170
(SEQ ID NO: 52)

In one aspect, the biological activity of the protein of the instant invention is that it can bind to and substantially inhibit the biological activity of trypsin, human plasma and tissue kallikreins, human plasmin and Factor XIIa. In a preferred embodiment, the present invention provides for a native human placental bikunin protein, in glycosylated form. In a further embodiment the instant invention encompasses native human bikunin protein which has been formed such that it contains at least one cysteine-cysteine disulfide bond. In a preferred embodiment, the protein contains at least one intra-chain cysteine-cysteine disulfide bond formed between a pair of cysteines selected from the group consisting of CYS11-CYS61, CYS20-CYS44, CYS36-CYS57, CYS106-CYS156, CYS115-CYS139, and CYS131-CYS152, wherein the cysteines are numbered according to the amino acid sequence of native human placental bikunin. One of ordinary skill will recognize that the protein of the instant invention may fold into the proper three-dimensional conformation, such that the biological activity of native human bikunin is maintained, where none, one or more, or all of the native intra-chain cysteine-cysteine disulfide bonds are present. In a most preferred embodiment, the protein of the instant invention is properly folded and is formed with all of the proper native cysteine-cysteine disulfide bonds.

Active protein of the instant invention can be obtained by purification from human tissue, such as placenta, or via synthetic protein chemistry techniques, as illustrated by the Examples below. It is also understood that the

protein of the instant invention may be obtained using molecular biology techniques, where self-replicating vectors are capable of expressing the protein of the instant invention from transformed cells. Such protein can be made as non-secreted, or secreted forms from transformed cells. In order to facilitate secretion from transformed cells, to enhance the functional stability of the translated protein, or to aid folding of the bikunin protein, certain signal peptide sequences may be added to the NH2-terminal portion of the native human bikunin protein.

In one embodiment, the instant invention thus provides for the native human bikunin protein with at least a portion of the native signal peptide sequence intact. Thus one embodiment of the invention provides for native human bikunin with at least part of the signal peptide, having the amino acid sequence:

```
AGSFLAWLGSLLLSGVLA                                   -1
ADRERSIHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN   50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF  100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE  150
ACMLRCFRQQENPPLPLGSKVVVLAGAVS                       179
```

(SEQ ID NO: 2)

In a prefered embodiment the instant invention provides for a native human placental bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with an intact leader segment having the amino acid sequence:

```
MAQLCGL RRSRAFLALL GSLLLSGVLA   -1
```

(SEQ ID NO: 53)

In another embodiment, the instant invention provides for bikunin protein with part of the leader sequence intact, having the amino acid sequence of SEQ ID NO: 52 with the intact leader segment having the amino acid sequence:

```
MLR AEADGVSRLL GSLLLSGVLA  -1
```

(SEQ ID NO: 54)

In a preferred numbering system used herein the amino acid numbered +1 is assigned to the NH2-terminus of the amino acid sequence for native

human placental bikunin. One will readily recognize that functional protein fragments can be derived from native human placental bikunin, which will maintain at least part of the biological activity of native human placental bikunin, and act as serine protease inhibitors.

In one embodiment, the protein of the instant invention comprises a fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 7-159, hereinafter called "bikunin (7-159)". Thus the instant invention embodies a protein having the amino acid sequence:

```
IHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN          50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF 100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRCFRQ                                          159
```

(SEQ ID NO: 3)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another functional variant of this embodiment can be the fragment of native human placental bikunin, which contains at least one functional Kunitz-like domain, having the amino acid sequence of native human placental bikunin amino acids 11-156, bikunin (11-156)

```
CLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN              50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDSEDHSSDMF 100
NYEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRC                                             156
```

(SEQ ID NO: 50).

One can recognize that the individual Kunitz-like domains are also fragments of the native placental bikunin. In particular, the instant invention provides for a protein having the amino acid sequence of a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 7-64, hereinafter called "bikunin (7-64)". Thus in one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

IHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKCATV 64
(SEQ ID NO: 4)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of the protein of the instant invention can be a first Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 11-61, "bikunin (11-61)" having the amino acid sequence:

CLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKC 61
(SEQ ID NO: 5)

The instant invention also provides for a protein having the amino acid sequence of a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 102-159, hereinafter called "bikunin (102-159)". Thus one embodiment the instant invention encompasses a protein which contains at least one Kunitz-like domain having the amino acid sequence:

YEEYCTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRCFRQ 159
(SEQ ID NO: 6)

where the amino acid numbering corresponds to that of the amino acid sequence of native human placental bikunin. Another form of this domain can be a Kunitz-like domain consisting of the amino acid sequence of native human placental bikunin amino acids 106-156, "bikunin (106-156)" having the amino acid sequence:

CTANAVTGPCRASFPRWYFDVERNSCNNFIYGGCRGNKNSYRSEE 150
ACMLRC 156
(SEQIDNO:7)

Thus one of ordinary skill will recognize that fragments of the native

human bikunin protein can be made which will retain at least some of the native protein biological activity. Such fragments can also be combined in different orientations or multiple combinations to provide for alternative proteins which retain some of, the same, or more biological activity of the native human bikunin protein.

One will readily recognize that biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional Kunitz-like domains from other sources. Biologically active protein of the instant invention may comprise one or more of the instant Kunitz-like domains in combination with additional protein domains from other sources with a variety of biological activities. The biological activity of the protein of the instant invention can be combined with that of other known protein or proteins to provide for multifunctional fusion proteins having predictable biological activity. Thus, in one embodiment, the instant invention encompasses a protein which contains at least one amino acid sequence segment the same as, or functionally equivalent to the amino acid sequence of either SEQ ID NO: 5 or SEQ ID NO: 7.

An open reading frame which terminates at an early stop codon can still code for a functional protein. The instant invention encompasses such alternative termination, and in one embodiment provides for a protein of the amino acid sequence:

ADRERSIHDFCLVSKVVGRCRASMPRWWYNVTDGSCQLFVYGGCDGNSNN 50
YLTKEECLKKCATVTENATGDLATSRNAADSSVPSAPRRQDS 92
(SEQIDNO:8)

In one embodiment, the instant invention provides for substantially purified, or recombinantly produced native human bikunin protein with an intact segment of the leader sequence, and at least a portion of the native transmembrane region intact. Thus one embodiment of the invention provides for native human bikunin, with an intact leader sequence, and with at least part of the transmembrane domain (underlined), having an amino acid sequence selected from:

```
1)EST                                 MLR AEADGVSRLL GSLLLSGVLA   -1
2)PCR                             MAQLCGL RRSRAFLALL GSLLLSGVLA   -1
3)λcDNA                           MAQLCGL RRSRAFLALL GSLLLSGVLA   -1

1)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN   50
2)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN   50
3)ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN   50

1)YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF  100
2)YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF  100
3)YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF  100

1)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE  150
2)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE  150
3)NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE  150

1)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN  200
2)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN  200
3)ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN  200

1)QERALRTVWS SGDDKEQLVK NTYVL                             225
2)QERALRTVWS FGD                                         213
3)QERALRTVWS SGDDKEQLVK NTYVL                             225
```

where sequence 1) is EST derived consensus SEQ ID NO: 45, 2) is PCR clone SEQ ID NO:47, and 3) is lambda cDNA clone SEQ ID NO:49. In a preferred embodiment a protein of the instant invention comprises one of the amino acid sequence of SEQ ID NO: 45, 47 or 49 wherein the protein has been cleaved in the region between the end of the last Kunitz domain and the transmembrane region.

The instant invention also embodies the protein wherein the signal peptide is deleted. Thus the instant invention provides for a protein having the amino acid sequence of SEQ ID NO: 52 continuous with a transmembrane amino acid sequence:

```
EST      VVVLAGLFVM VLILFLGASM VYLIRVARRN      200
EST      QERALRTVWS SGDDKEQLVK NTYVL           225
         (SEQ ID NO: 69)
```

a transmembrane amino acid sequence:

```
PCR      VVVLAGLFVM VLILFLGASM VYLIRVARRN      200
PCR      QERALRTVWS FGD                        213
         (SEQ ID NO: 68)
```

or a transmembrane amino acid sequence:

```
λcDNA    VVVLAGLFVM VLILFLGASM VYLIRVARRN      200
λcDNA    QERALRTVWS SGDDKEQLVK NTYVL           225
         (SEQ ID NO: 67).
```

The protein amino acid sequences of the instant invention clearly teach one of the art the appropriate nucleic acid sequences which can be used in molecular biology techniques to produce the proteins of the instant invention. Thus, one embodiment of the instant invention provides for a nucleic acid sequence which encodes for a human bikunin having the consensus DNA sequence of Figure 3 (SEQ ID NO: 9), which translates into the amino acid sequence for native human placental bikunin sequence of Figure 3 (SEQ ID NO: 10). In another embodiment, the instant invention provides for a consensus nucleic acid sequence of Figure 4C (SEQ ID NO: 51) which encodes for an amino acid sequence of Figure 4D (SEQ ID NO: 45).

In a preferred embodiment, the instant invention provides for a nucleic acid sequence which encodes for native human placental bikunin having the DNA sequence of Figure 4F (SEQ ID NO: 48) which encodes for the protein sequence of SEQ ID NO: 49. In an another embodiment, the instant invention provides for a nucleic acid sequence of Figure 4E (SEQ ID NO: 46) which encodes for a protein sequence of SEQ ID NO: 47.

One can easily recognize that certain allelic mutations, and conservative substitutions made in the nucleic acid sequence can be made which will still result in a protein amino acid sequence encompassed by the instant invention. One of skill in the art can recognize that certain natural allelic mutations of the protein of the instant invention, and conservative substitutions of amino acids in the protein of the instant invention will not significantly alter the biological activity of the protein, and are encompassed by the instant invention.

The instant invention also provides for pharmaceutical compositions containing human placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery.

The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention in a biologically compatible vehicle is administered to the patient.

The present invention also provides for variants of placental bikunin, and the specific Kunitz domains described above, that contain amino acid substitutions that alter the protease specificity. Preferred sites of substitution are indicated below as positions Xaa^1 through Xaa^{32} in the amino acid sequence for native placental bikunin. Substitutions at Xaa^1 through Xaa^{16} are also preferred for variants of bikunin (7-64), while substitutions at Xaa^{17} through Xaa^{32} are preferred for variants of bikunin (102-159).

Thus the present invention embodies protein having an amino acid sequence:

Ala Asp Arg Glu Arg Ser Ile Xaa^{1} Asp Phe 10
Cys Leu Val Ser Lys Val Xaa^{2} Gly Xaa^{3} Cys 20
Xaa^{4} Xaa^{5} Xaa^{6} Xaa^{7} Xaa^{8} Xaa^{9} Trp Trp Tyr Asn 30
Val Thr Asp Gly Ser Cys Gln Leu Phe Xaa^{10} 40
Tyr Xaa^{11} Gly Cys Xaa^{12} Xaa^{13} Xaa^{14} Ser Asn Asn 50
Tyr Xaa^{15} Thr Lys Glu Glu Cys Leu Lys Lys 60
Cys Ala Thr Xaa^{16} Thr Glu Asn Ala Thr Gly 70
Asp Leu Ser Thr Ser Arg Asn Ala Ala Asp 80
Ser Ser Val Pro Ser Ala Pro Arg Arg Gln 90
Asp Ser Glu His Asp Ser Ser Asp Met Phe 100
Asn Tyr Xaa^{17} Glu Tyr Cys Thr Ala Asn Ala 110
Val Xaa^{18} Gly Xaa^{19} Cys Xaa^{20} Xaa^{21} Xaa^{22} Xaa^{23} Xaa^{24} 120
Xaa^{25} Trp Tyr Phe Asp Val Glu Arg Asn Ser 130
Cys Asn Asn Phe Xaa^{26} Tyr Xaa^{27} Gly Cys Xaa^{28} 140
Xaa^{29} Xaa^{30} Lys Asn Ser Tyr Xaa^{31} Ser Glu Glu 150
Ala Cys Met Leu Arg Cys Phe Arg Xaa^{32} Gln 160
Glu Asn Pro Pro Leu Pro Leu Gly Ser Lys 170
Val Val Val Leu Ala Gly Ala Val Ser 179
(SEQ ID NO: 11).

where Xaa^{1} - Xaa^{32} each independently represents a naturally occurring amino acid residue except Cys, with the proviso that at least one of the amino acid residues Xaa^{1}-Xaa^{32} is different from the corresponding amino acid residue of the native sequence.

In the present context, the term "naturally occurring amino acid residue" is intended to indicate any one of the 20 commonly occurring amino acids, i.e., Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

By substituting one or more amino acids in one or more of the positions indicated above, it may be possible to change the inhibitor specificity profile of native placental bikunin or that of the individual Kunitz-like domains, bikunin(7-64) or bikunin (102-159) so that it preferentially inhibits other serine proteases such as, but not limited to, the enzymes of the complement cascade, TF/FVIIa, FXa, thrombin, neutrophil elastase, cathepsin G or proteinase-3.

Examples of preferred variants of placental bikunin include those

wherein Xaa^1 is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Val or Lys, in particular wherein Xaa^1 is His or Pro; or wherein Xaa^2 is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala, Lys, in particular wherein Xaa^2 is Val or Thr; or wherein Xaa^3 is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu, Thr, in particular wherein Xaa^3 is Arg or Pro; or wherein Xaa^4 is an amino acid residue selected from the group consisting of Arg, Lys and Ser, Gln, in particular wherein Xaa^4 is Arg or Lys; or wherein Xaa^5 is an amino acid residue selected from the group consisting of Ala, Gly, Asp, Thr, in particular wherein Xaa^5 is Ala; or wherein Xaa^6 is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg, Phe, in particular wherein Xaa^6 is Ser or Arg ; or wherein Xaa^7 is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa^7 is Met or Ile; or wherein Xaa^8 is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Gln, Asn, Leu, Ser or Ile, in particular wherein Xaa^8 is Pro or Ile; or wherein Xaa^9 is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa^9 is Arg: or wherein Xaa^{10} is an amino acid residue selected from the group consisting of Val, Ile, Lys, Ala, Pro, Phe, Trp, Gln, Leu and Thr, in particular wherein Xaa^{10} is Val; or wherein Xaa^{11} is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa^{11} is Gly; or wherein Xaa^{12} is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gln, Gly, in particular wherein Xaa^{12} is Asp or Arg; or wherein Xaa^{13} is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa^{14} is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa^{15} is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa^{15} is Leu or Lys; or wherein Xaa^{16} is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Val, in particular wherein Xaa^{16} is Val or Ala; or wherein Xaa^{17} is an amino acid residue selected from the group consisting of His, Glu, Pro, Ala, Lys and Val, in particular wherein Xaa^{17} is Glu or Pro; or wherein Xaa^{18} is an amino acid residue selected from the group consisting of Val, Thr, Asp, Pro, Arg, Tyr, Glu, Ala or Lys, in particular wherein Xaa^{18} is Thr; or wherein Xaa^{19} is an amino acid residue selected from the group consisting of Arg, Pro, Ile, Leu or Thr, in particular wherein Xaa^{19} is Pro; or wherein Xaa^{20} is an amino acid residue selected from the group consisting of Arg, Lys, Gln and

Ser, in particular wherein Xaa^{20} is Arg or Lys; or wherein Xaa^{21} is an amino acid residue selected from the group consisting of Ala, Asp, Thr or Gly ; in particular wherein Xaa^{21} is Ala; or wherein Xaa^{22} is an amino acid residue selected from the group consisting of Ser, Ile, Tyr, Asn, Leu, Val, Arg or Phe, in particular wherein Xaa^{22} is Ser or Arg ; or wherein Xaa^{23} is an amino acid residue selected from the group consisting of Met, Phe, Ile, Glu, Leu, Thr and Val, in particular wherein Xaa^{23} is Phe or Ile; or wherein Xaa^{24} is an amino acid residue selected from the group consisting of Pro, Lys, Thr, Asn, Leu, Gln, Ser or Ile, in particular wherein Xaa^{24} is Pro or Ile; or wherein Xaa^{25} is an amino acid residue selected from the group consisting of Arg, Lys or Leu, in particular wherein Xaa^{25} is Arg: or wherein Xaa^{26} is an amino acid residue selected from the group consisting of Val, Ile, Lys, Leu, Ala, Pro, Phe, Gln, Trp and Thr, in particular wherein Xaa^{26} is Val or Ile; or wherein Xaa^{27} is an amino acid residue selected from the group consisting of Gly, Ser and Thr, in particular wherein Xaa^{27} is Gly; or wherein Xaa^{28} is an amino acid residue selected from the group consisting of Asp, Arg, Glu, Leu, Gly or Gln, in particular wherein Xaa^{28} is Arg; or wherein Xaa^{29} is an amino acid residue selected from the group consisting of Gly and Ala; or wherein Xaa^{30} is an amino acid residue selected from the group consisting of Asn or Lys; or wherein Xaa^{31} is an amino acid residue selected from the group consisting of Gly, Asp, Leu, Arg, Glu, Thr, Tyr, Val, and Lys, in particular wherein Xaa^{31} is Arg or Lys; or wherein Xaa^{32} is an amino acid residue selected from the group consisting of Val, Gln, Asp, Gly, Ile, Ala, Met, and Thr, in particular wherein Xaa^{32} is Gln or Ala.

Description of the Drawings

The invention will be better understood from a consideration of the following detailed description and claims, taken in conjunction with the drawings, in which:

Figure 1 depicts the nucleotide sequence of EST R35464 (SEQ ID NO: 12) and the translation of this DNA sequence (SEQ ID NO: 13) which yielded an open reading frame with some sequence similarity to aprotinin. The translation product contains 5 of the 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). The position normally occupied by the remaining cysteine (at codon 38) contained instead a phenylalanine (indicated by an asterisk).

Figure 2 depicts the nucleotide sequence of EST R74593 (SEQ ID NO: 14),

and the translation of this DNA sequence (SEQ ID NO: 15) which yielded an open reading frame with homology to the Kunitz class of serine protease inhibitor domains. The translation product contained 6 cysteines in the correct spacing that is characteristic for Kunitz-like inhibitor domains (indicated in bold). However, this reading frame sequence includes stop codons at codon 3 and 23.

Figure 3 depicts a deduced nucleic acid sequence of human placental bikunin (SEQ ID NO: 9) labeled "consensus" and matched with the translated protein amino acid sequence labeled "translated" (SEQ ID NO: 10). Also as comparison are shown the nucleic acid sequence for ESTs H94519 (SEQ ID NO: 16), N39798 (SEQ ID NO: 17), R74593 (SEQ ID NO: 14) and R35464 (SEQ ID NO: 12). The underlined nucleotides in the consensus sequence correspond to the site of PCR primers described in the Examples. Underlined amino acids in the translated consensus sequence are residues whose identity have been confirmed by amino acid sequencing of purified native human placental bikunin. Nucleotide and amino acid code are standard single letter code, "N" in the nucleic acid code indicates an unassigned nucleic acid, and "*" indicates a stop codon in the amino acid sequence.

Figure 4A depicts the original overlay of a series of ESTs with some nucleic acid sequence homology to ESTs encoding human placental bikunin, or portions thereof. Shown for reference are the relative positions of bikunin (7-64) and bikunin (102-159), labeled KID1 and KID2 respectively.

Figure 4B depicts a subsequent more comprehensive EST overlay incorporating additional ESTs. Numbers on the upper X-axis refer to length in base pairs, starting at the first base from the most 5' EST sequence. The length of each bar is in proportion to the length in base pairs of the individual ESTs including gaps. The EST accession numbers are indicated to the right of their respective EST bars.

Figure 4C depicts the corresponding alignment of the oligonucleotide sequences of each of the overlapping ESTs shown schematically in Figure 4B. The upper sequence (SEQ ID NO: 51) labeled bikunin represents the consensus oligonucleotide sequence derived from the overlapping nucleotides at each position. The numbers refer to base-pair position within the EST map. The oligonucleotides in EST R74593 that are bold underlined (at map positions 994 and 1005) are base insertions observed in R74593 that were consistently absent in each of the other overlapping ESTs.

Figure 4D depicts the amino acid translation of the consensus

oligonucleotide sequence for bikunin depicted in Figure 4C (SEQ ID NO: 45).

Figure 4E depicts the nucleotide sequence (SEQ ID NO: 46) and corresponding amino acid translation (SEQ ID NO: 47) of a placental bikunin encoding sequence that was derived from a human placental cDNA library by PCR-based amplification.

Figure 4F depicts the nucleotide sequence (SEQ ID NO: 48) and corresponding amino acid translation (SEQ ID NO: 49) of a native human placental bikunin encoding clone that was isolated from a human placental lambda cDNA library by colony hybridization.

Figure 4G compares the alignment of the amino acid translated oligonucleotide sequences for placental bikunin obtained by EST overlay (SEQ ID NO: 45), PCR based cloning (SEQ ID NO: 47), and conventional lambda colony hybridization (SEQ ID NO: 49).

Figure 5 shows a graph of purification of human placental bikunin from placental tissue after Superdex 75 Gel-Filtration. The plot is an overlay of the protein elution profile as measured by OD 280 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 6 shows a graph which plots the purification of human placental bikunin from placental tissue using C18 Reverse-Phase Chromatography. The plot is an overlay of the protein elution profile as measured by OD 215 nm (solid line), activity of eluted protein in a trypsin inhibition assay (% inhibition shown by circles), and activity of eluted protein in a kallikrein inhibition assay (% inhibition shown by squares).

Figure 7 depicts a silver stained SDS-PAGE gel of highly purified placental bikunin (lane 2), and a series of molecular size marker proteins (lane 1) of the indicated sizes in kilodaltons. Migration was from top to bottom.

Figure 8 shows the amount of trypsin inhibitory activity present in the cell-free fermentation broth from the growth of yeast strains SC101 (panel 8A) or WHL341 (panel 8B) that were stably transformed with a plasmid (pS604) that directs the expression of placental bikunin (102-159).

Figure 9 shows both a silver stained SDS-PAGE (left panel) and a Western blot with anti-placental bikunin (102-159) pAb (right panel) of cell-free fermentation broth from the growth of yeast strain SC101 (recombinants 2.4 and 2.5) that was stably transformed with a plasmid directing the expression of either bovine aprotinin, or placental bikunin (102-159). Migration was from top

to bottom.

Figure 10 is a photograph which shows a silver stained SDS-PAGE of highly purified placental bikunin (102-159) (lane 2) and a series of molecular size marker proteins (lane 1) of the indicated sizes in Kilodaltons. Migration was from top to bottom.

Figure 11 is a photograph which shows the results of Northern blots of mRNA from various human tissues that was hybridized to a ^{32}P labeled cDNA probe encoding either placental bikunin (102-159) (panel 11A) or encoding placental bikunin (1-213) (panel 11B). Migration was from top to bottom. The numbers to the right of each blot refer to the size in kilobases of the adjacent RNA markers. The organs from which mRNA was derived is described under each lane of the blot.

Figure 12 depicts an immunoblot of placental derived placental bikunin with rabbit antiserum raised against either synthetic reduced placental bikunin (7-64) (panel A) or 102-159 (panel B). For each panel, contents were: molecular size markers (lanes 1); native placental bikunin isolated from human placenta (lanes 2); synthetic placental bikunin (7-64) (lanes 3) and synthetic placental bikunin (102-159) (lanes 4). Tricine 10-20% SDS-PAGE gels were blotted and developed with protein A-purified primary polyclonal antibody (8 ug IgG in 20 ml 0.1% BSA/Tris-buffered saline (pH 7.5), followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody. Migration was from top to bottom.

Figure 13 depicts a Coomassie Blue stained 10-20% Tricine SDS-PAGE gel of 3 micrograms of highly purified placental bikunin (1-170) derived from a baculovirus / Sf9 expression system (lane 2). Lane 1 contains molecular size markers. Migration was from top to bottom.

Figure 14 depicts a comparison of the effect of increasing concentrations of either Sf9-derived human placental bikunin (1-170) (filled circles), synthetic placental bikunin (102-159) (open circles), or aprotinin (open squares) on the activated partial thromboplastin time of human plasma. Clotting was initiated with $CaCl_2$. The concentration of proteins are plotted versus the -fold prolongation in clotting time. The uninhibited clotting time was 30.8 seconds.

Detailed Description of the Invention

The present invention encompasses a newly identified human protein herein called human placental bikunin that contains two serine protease inhibitor domains of the Kunitz class. The instant invention also encompasses

pharmaceutical compositions containing placental bikunin and fragments thereof that are useful for the reduction of perioperative blood loss in a patient undergoing surgery, or with major trauma.

The present invention also provides methods for reducing perioperative blood loss in a patient undergoing surgery or due to major trauma, wherein an effective amount of the disclosed human serine protease inhibitors of the present invention, in a biologically compatible vehicle, is administered to the patient.

A preferred application for placental bikunin, isolated domains, and other variants is for the reduction of blood loss resulting from trauma or surgery that has the potential for loss of large volumes of blood. These methods and compositions reduce or eliminate the need for whole donor blood or blood products, thereby reducing the risk of infection and other adverse side effects , as well as the cost of surgery. The methods are thus useful in reducing blood loss in normal patients, i.e., those not suffering from inborn or other pre-operative deficiencies in coagulation factors. The reduction in blood loss is seen as a reduction in blood loss during surgery , as reduced post surgical drainage or both. Preferred surgical applications include but are not limited to use in thoracic and abdominal surgery, total and partial hip replacement surgeries and surgeries to treat a patient having an epithelial lesion of the eye. Preferred thoracic surgical procedures include but are not limited to aortocoronary bypass, excision of cardiac and aortic aneurysms, and surgery for esophageal varices, and coronary artery bypass surgery. Preferred abdominal surgeries include but are not limited to liver transplants, radical prostatectomy, surgery for diverticulitis of colon, tumor debulking, surgery on the abdominal aorta and surgery for duodenal ulcers, and repair of liver or spleen trauma. Preferred use for the treatment of trauma include but are not limited to the use in stabilization of severely injured patients at accident sites suffering from e.g., limb loss or major thoracic /abdominal wounds. In case of use for the reduction of blood loss resulting from surgery it is preferred to administer the placental bikunin, isolated domains, or other variant prior to and during surgery, whereas in case of use in trauma settings the placental bikunin variant, isolated domain or other variant is to be administered as rapidly as possible following injury, and should be contained on emergency vehicles traveling to the accident sites.

Factor XII (also known as Hageman Factor) is a serine protease that is found in the circulation in a zymogen form (80 kD) at approximately 29-40

μg/ml (see Pixley, et al. (1993) *Meth. in Enz.*, 222, 51-64) and is activated by tissue and plasma kallikrein. Once activated, it participates in the intrinsic pathway of blood coagulation which is activated when blood or plasma contacts a "foreign" or anionic surface. Once activated, Factor XIIa can then cleave and activate a number of other plasma proteases including Factor XI, prekallikrein, and C1 of the complement system. Thus Factor XII may be involved in causing hypotensive reactions since activated kallikrein can cleave kininogen releasing bradykinin (see Colman, (1984) *J. Clin. Invest.*, 73, 1249).

Sepsis is a disease that results from bacterial infection due to bacterial endotoxin or lipopolysaccharide (LPS). Exposure of Factor XII to LPS results in the activation of Factor XII. Patients with sepsis frequently have symptoms of intravascular coagulation which may also be due to activation of Factor XII by LPS. Septic shock can result from bacterial infection and is associated with fever, low systemic vascular resistance, and low arterial pressure. It is a common cause of death in intensive care units in the United States, where seventy five percent of the patients that die from septic shock have a persistent hypotension (see Parillo, et al. (1989) *Ann Rev. Med.*, 40, 469-485).

Adult respiratory distress syndrome is characterized by pulmonary edema, hypoxemia, and decreased pulmonary compliance. The pathogenesis of the disease is currently unknown although the proteolytic pathways of coagulation and fibrinolysis are believed to play a role (see Carvalho, et al. (1988) *J. Lab Clin. Med.*, 112: 270-277).

The proteins of the instant invention are also a novel human Kunitz type inhibitor of kallikrein, an activator of Factor XII. Thus another object of the current invention is to present a method for the prophylactic or therapeutic treatment of systemic inflammatory reactions such as septic shock, adult respiratory distress syndrome (ARDS), preeclampsia, multiple organ failure and disseminated intravascular coagulation (DIC). The therapeutic or prophylactic administration of the peptides of the instant invention would result in the modulation of these inflammatory conditions and be beneficial to the patient.

Plasmin plays an important role in extracellular matrix degradation and the activation of matrix-metallo protease (MMP) cascades. Collectively these proteases mediate migration of and tissue invasion by both endothelial cells during angiogenesis/neovascularization, and cancer cells during metastasis. Neovascularization is essential to support tumor growth and metastasis is a process which mediates the spreading of tumors and which is associated with

extremely poor patient prognosis.

Several preclinical studies suggest that Kunitz like serine protease inhibitors with a protease specificity similar to aprotinin are useful as medicaments for cancer. For example, aprotinin reduced tumor growth and invasion, with increased tumor necrosis when administered to hamsters bearing a highly invasive fibrosarcoma or to mice bearing a similarly malignant mammary carcinoma (Latner et al., (1974), Br. J. Cancer 30: 60-67; Latner and Turner, (1976), Br. J. Cancer 33: 535-538). Furthermore, administration of 200,000 KIU of aprotinin i.p. to C57B1/6 Cr male mice on days 1 to 14 post-inoculation with Lewis lung carcinoma cells, reduced pulmonary metastases by 50% although had no effect on primary tumor mass (Giraldi et al., (1977) Eur. J. Cancer, 13: 1321-1323). Similarly, administration of 10,000 KIU i.p. on each of days 13-16 post-inoculation of C57BL/6J mice with Lewis tumor cells inhibited pulmonary metastases by 90% without affecting the primary tumor growth (Uetsuji et al., (1992), Jpn. J. Surg. 22: 429-442). In this same study, administration of plasmin or kallikrein with the same dosing schedule was argued to increase the number of pulmonary metastases. These results prompted the authors to suggest that perioperative administration of aprotinin to cancer patients may reduce the likelihood of metastases. Black and Steger (1976, Eur. J. Pharmacol., 38: 313-319) found that aprotinin inhibited the growth of the transplanted rodent Murphy-Strum lymphosarcoma in rats and suggested that the effect involved the inhibition of the kinin-forming enzyme system. Twice daily i.p. injection of female ddY mice with 10,000 KIU of aprotinin for 7 weeks to mice each bearing a single autochtonous squamous cell carcinoma resulting from 3-methylcholanthrene treatment reduced the growth rate of the primary tumors by 90%. In some animals tumor regression was observed. While all vehicle treated animals had died within the seven weeks, all of the aprotinin treatment group remained alive. Reduced tumor growth was associated with hyperkeratosis (Ohkoshi, Gann (1980), 71: 246-250).

Clinically, a surgically cured group of 26 patients who received aprotinin i.v. exhibited a 70% survival two years post surgery with no recurrence of tumors whereas a placebo group of 26 patients at the same time exhibited only a 38% survival with a significant rate of tumor recurrence (Freeman et al. Br. Soc. Gastroenterol. (1980) supplement A: 902). In a case study (Guthrie et al., Br. J. Clin. Pract (1981) 35: 330-332), administration of bromocriptine plus aprotinin to a patient with advanced cancer of the cervix caused remission. Aprotinin was administerd both as a 500,000 KIU i.p. bolus every eight hours concurrently

with a continuous i.v. infusion of aprotinin at a rate of 200,000 KIU per 6 hr for a total of seven days once a month. Treatment was ended at the end of the fourth month due to the development of an allergic reaction to aprotinin. More recent evidence has further underscored a role of plasmin as a target for these effects of aprotinin on metastases.

The mechanism for these events could be related to the fact that aprotinin blocks the invasive potential of cancer cell lines (Liu G., et al., Int J. Cancer (1995), 60: 501-506). Furthermore, since the proteins of the instant invention are also potent inhibitors of plasmin and kallikrien, they are contemplated for use as anti-cancer agents. For example they are contemplated for use in blocking primary tumor growth by restricting neovascularization, primary tumor invasion and in blocking metastasis through inhibition of tissue infiltration. The compounds may be administered locally to tumors or systemically. In a preferred mode of treatment, the protein would be administered perioperatively during tumor debulking to minimize the risk of metastasis. In such a regime, the blood sparing properties of the compound would be additionally advantageous in providing a clearer surgical field of view. Another preferred mode of administration would be as a combination therapy with either MMP inhibitors or chemotherapy. An additional preferred mode of administration would be as a locally administered gene therapy designed to achieve selective expression of placental bikunin within the tumor cells, or their associated stroma and vascular beds.

Preferred types of cancers targeted for therapy would be vasular-dependent solid tumors such as breast, colon, lung, prostate and ovarian carcinomas which exhibit a high metastatic potential, and those for which local delivery of a high concentration of the protein is feasible such as lung cancers through pulmonary delivery, colon carcinomas through hepatic delivery to liver metastasis, or skin cancers such as head and neck carcinomas or melanomas through subcutaneous delivery. Since the proteins of the present invention are of human origin they would be less likely to be associated with allergic or anaphylactic reactions of the kind observed by Guthrie et al., *supra*, upon reuse.

Additionally, the proteins of the present invention are contemplated for use in the reduction of thromboembolic complications associated with activation of the intrinsic pathway of coagulation. This would include prevention of pulmonary embolism in late stage cancer patients, a frequent cause of death (Donati MB., (1994), Haemostasis 24: 128-131).

Edema of the brain and spinal cord is a complication resulting from traumatic brain or spinal cord injury, stroke, cerebral ischemia, cerebral and sub-arachnoid hemhorrhage, surgery (including open heart surgery), infectious diseases such as encephalitis and meningitis, granulomatous diseases such as Sarcoid and focal or diffuse carcinomas, and is a contributor to the high level of morbidity and death following these events. Bradykinin is known to disrupt the blood brain barrier experimentally (Greenwood J., (1991), Neuroradiology, 33: 95-100; Whittle et al., (1992), Acta Neurochir., 115: 53-59), and infusion of bradykinin into the internal carotid artery induced brain edema in spontaneously hypertensive rats (SHR) subjected to common carotid artery occlusion (Kamiya, (1990), Nippon Ika Daigaku Zasshi. 57: 180-191). Elevated levels of bradykinin are found in extracellular fluids following trauma in a model involving traumatized rat spinal chord (Xu et al., (1991), J. Neurochem, 57: 975-980), and in plasma and tissue from rats with brain edema resulting from cerebral ischaemia (Kamiya et al., (1993), Stroke, 24: 571-575). Bradykinin is released from high molecular weight kininogen by serine proteases including kallikrein (Coleman (1984) J. Clin Invest., 73: 1249), and the serine protease inhibitor aprotinin was found to block the magnitude of brain edema resulting from cerebralschemia in SHR rats (Kamiya, (1990), Nippon Ika Daigaku Zasshi. 57: 180-191; Kamiya et al., (1993), Stroke, 24: 571-575) and rabbits subjected to a cold lesion of the brain (Unterberg et al., (1986), J. Neurosurgery, 64: 269-276).

These observations indicate that brain edema results from local proteolytic release of kinins such as bradykinin from high molecular weight kininogen, followed by bradykinin-induced increases in blood brain barrier permeability. Accordingly, placental bikunin and fragments thereof are contemplated as medicaments for the prevention of edema in patients at risk for this condition, particularly those of high risk of mortality or brain injury. This would include head and spinal trauma patients, polytrauma patients, patients undergoing surgery of the brain or spinal cord and their associated vessels or other generalsurgeries including open-heart surgery, patients who have suffered from a stroke, cerebral or sub-arachnoid hemorrhage, infectious diseases of the brain, granulomatous disease of the brain or diffuse or focal carcinomas and tumors of the brain or any conditions such as multiple sclerosis involving breakdown of the blood brain barrier or patients suffering from any other inflammatory processes of the brain or spinal cord. Patients would receive an administration of placental bikunin either as an infusion or bolus injection, intravenously or intracranially. Additional doses of placental bikunin

could be administered intermittently over the following one to three weeks. Dose levels would be designed to attain circulating concentrations in excess of those required to neutralize elevations in plasma levels or bradykinin and other vasoactive peptides formed through the action of serine proteases, and sufficient to reduce edema. Since the protein is of human origin, repeated administration in this course of therapy would not lead to development of an immune reaction to the protein. Placental bikunin and fragments thereof would be contemplated for monotherapy or prophylacsis as well as for use in combination with other medicaments such as neurotherapeutics and neuroprotectants.

Recent evidence (Dela Cadena R. A. at al., (1995), FASEB J. 9: 446-452) has indicated that the contact activation pathway may contribute to the pathogenesis of arthritis and anemia, and that kallikrein inhibitors may be of therapeutic benefit. Accordingly, protease inhibitors of the present invention are contemplated according to their capacity to inhibit human kallikrein, as medicaments for the treatment of arthritis and anemia in humans.

Treatment of male non-insulin diabetic (NIDDM) patients with aprotinin significantly improved total glucose uptake and decreased the metabolic clearance rate of insulin (Laurenti et al., (1996), Diabetic Medicine 13: 642-645). Accordingly, the human proteins of the present invention are contemplated for chronic use as medicaments for the treatment of NIDDM.

Daily treatment of patients at risk of preterm delivery with urinary trypsin inhibitor for two weeks significantly reduced recurrent uterine contractions (Kanayama et al., (1996), Eur J. Obstet. Gynecol. & Reprod. Biol. 67: 133-138). Accordingly, the human proteins of the present invention are contemplated for use in the prevention of preterm delivery.

Aprotinin has been shown to stimulate differentiation of mouse myoblasts in culture (Wells and Strickland, Development, (1994), 120: 3639-3647)), a process that is inhibited by TGFb. TGFb exists as an inactive pro-polypeptide which is activated by limited proteolysis. The mechanism of aprotinin action has been proposed to involve inhibition of proteases which process pro-TGFb to the mature active form. TGFb has been shown to be up-regulated in various fibrotic lesions and has long thought to be a potential target for anti-fibrotic therapies. In a rat model of pulmonary fibrosis for example, TGF-b concentrations paralleled the extent of bleomycin-induced inflammation. Furthermore, plasmin levels in the alveolar macrophage coincided with mature TGF-b levels, and the addition of the plasmin inhibitor

a-2-antiplasmin abrogated the post translational activation of pro-TGFb by the macrophage (Khal et al., (1996), Am. J. Respir. Cell Mol. Biol. 15: 252-259.) The data suggest that plasmin contributes to the formation of active TGFb by alveolar macrophage, and that this process plays a pathologic role in the bleomycin-induced lung inflammation.

In light of these observations, placental bikunin and fragments thereof are contemplated as therapeutics for various fibrotic disorders, including pulmonary, hepatic, renal and dermal (scleroderma) fibrosis.

Aerosilized aprotinin was shown to protect >50% of mice infected with lethal doses of either influenza virus or paramyxovirus (Ovcharenko and Zhirnov, Antiviral Research, (1994), 23: 107-118). A suppression of the development of fatal hemorrhagic bronchopneumonia and a normalization of body weight gain were also noted with aerosilized aprotinin treatment. In light of these observations, placental bikunin and fragments thereof are contemplated as therapeutics for various respiratory related influenza-like diseases.

The human placental bikunin, isolated domains, and other variants of the invention are contemplated for use in the medical/therapeutic applications suggested for native aprotinin or aprotinin analogues with other inhibitory profiles, in particular those which necessitate usage of large doses. These would include diseases for which use of the human protein is indicated by virtue of its ability to inhibit human serine proteases such as trypsin, plasmin, kallikrein, elastase, cathepsin G and proteinase-3, which include and are not limited to: acute pancreatitis (pancreatic elastase and trypsin), inflammation, thrombocytopenia, preservation of platelet function, organ preservation, wound healing, various forms of shock, including shock lung, endotoxin shock and post operative complications; disturbances of blood coagulation such as hyperfibrinolytic hemorrhage; acute and chronic inflammatory reactions, in particular for the therapy and prophylaxis of organ lesions, such as for example pancreatitis and radiation induced enteritis, complex-mediated inflammatory reactions such as immunovasculitis, glomerulonephritis and types of arthritis; collagenoses in particular rheumatoid arthritis; types of arthritis caused by metabolism-related deposits (for example gout); degeneration of the elastic constituents of the connective tissue parts of organs, such as in atherosclerosis (serum elastase) or pulmonary emphysema (neutrophil elastase); adult respiratory distress syndrome, inflammatory bowel disease, and psoriasis.

A major unexpected finding was that the synthetic peptides encoding

bikunin (7-64), and bikunin (102-159), could properly fold into the correct three-dimensional conformation having active protease inhibitor bioactivity (Examples 2 and 1, respectively). Upon folding, each of these fragments of Bikunin underwent a reduction in mass of 6 mass units, consistent with the formation in each case, of three intrachain disulfide bonds between six cysteine residues of each fragment. Another surprising finding is that the synthetic peptides encoding bikunin (7-64), bikunin (102-159), and bikunin (1-170) are highly inhibitory of plasmin and both tissue and plasma kallikrein (Example 4 , 3, and 10 respectively). Inhibition of plasmin and kallikrein by Trasylol® is thought to be involved in the mechanism by which Trasylol® reduces blood loss during open heart surgery. Our unexpected findings of the specificity of the Kunitz domains of the present invention make them suitable therapeutic agents for blood sparing during surgery or trauma where there is significant blood loss, or for any other condition where inhibition of plasmin and/or kallikrein would be beneficial.

Furthermore, we showed in this disclosure (Example 10) that placental bikunin (1-170) is a potent inhibitor of factor XIa and a moderate inhibitor of factor Xa. Factor XIa plays an essential role in the intrinsic pathway of coagulation, serving to interconvert inactive factor IX into active factor IXa. Thus, Placental Bikunin inhibits two key enzymes of the intrinsic pathway, kallikrein and factor XIa. Consistent with these observations, we also showed that placental bikunin (1-170) is a potent inhibitor of the activated partial thromboplastin time, which is a measure of the speed of coagulation driven by the intrinsic pathway. On the other hand, we showed that Placental bikunin (1-170) is an extremely weak inhibitor of the tissue factor VIIa complex, suggesting that it is not important in the regulation of the extrinsic coagulation cascade. Based on these unexpected findings, placental bikunin is contemplated as a medicament for diseases in which activation of the intrinsic pathway of coagulation contributes significantly to the disease mechanism. Examples of such diseases would include post-traumatic shock and disseminated intravascular coagulation.

A significant advantage of the Kunitz domains of the present invention is that they are human proteins, and also less positively charged than Trasylol® (Example 1), thereby reducing the risk of kidney damage on administration of large doses of the proteins. Being of human origin, the protein of the instant invention can thus be administered to human patients with significantly reduced risk of undesired immunological reactions as compared to

administration of similar doses of Trasylol®. Furthermore, it was found that bikunin (102-159), bikunin (7-64), and bikunin (1-170) are significantly more potent inhibitors of plasma kallikrein than Trasylol® *in vitro* (Example 3, 4 and 10). Thus bikunin and fragments thereof are expected to be more effective *in vivo* at lowering blood loss in patients.

The amount of serine protease inhibitor administered should be sufficient to provide a supra normal plasma level. For the prophylactic reduction of bleeding during and following coronary aortic by-pass surgery (CABG), the proteins of the instant invention may be used in place of Trasylol® while taking into account the differences in potency. The use of Trasylol® is outlined in the Physicians Desk Reference, (1995), listing for Trasylol® supplement A. Briefly, with the patient in a supine position, the loading dose of placental bikunin, isolated domain or other variant is given slowly over about 20 to 30 minutes, after induction of anesthesia but prior to sternotomy. In general, a total dose of between about $2x10^6$ KIU (kallikrein inhibitory units) and $8 X10^6$ KIU will be used, depending on such factors as patient weight and the length of the surgery. Preferred loading doses are those that contain a total of 1 to 2 million kallikrein inhibitory units (KIU). When the loading dose is complete, it is followed by the constant infusion dose, which is continued until surgery is complete and the patient leaves the operating room. Preferred constant infusion doses are in the range of about 250,000 to 500,000 KIU per hour. The pump prime dose is added to the priming fluid of the cardiopulmonary bypass circuit, by replacement of an aliquot of the priming fluid prior to the institution of the cardiopulmonary bypass. Preferred pump prime doses are those that contain a total of about one to two million KIU.

The proteins of the instant invention are employed in pharmaceutical compositions formulated in the manner known to the art. Such compositions contain active ingredient(s) plus one or more pharmaceutically acceptable carriers, diluents, fillers, binders, and other excipients, depending on the administration mode and dosage form contemplated. Examples of therapeutically inert inorganic or organic carriers known to those skilled in the art include, but are not limited to, lactose, corn starch or derivatives thereof, talc, vegetable oils, waxes, fats, polyols such as polyethylene glycol, water, saccharose, alcohols, glycerin and the like. Various preservatives, emulsifiers, dispersants, flavorants, wetting agents, antioxidants, sweeteners, colorants, stabilizers, salts, buffers and the like can also be added, as required to assist in the stabilization of the formulation or to assist in increasing bioavailability of

the active ingredient(s) or to yield a formulation of acceptable flavor or odor in the case of oral dosing. The inhibitor employed in such compositions may be in the form of the original compound itself, or optionally, in the form of a pharmaceutically acceptable salt. The proteins of the instant invention can be adminstered alone, or in various combinations, and in combination with other therapeutic compositions. The compositions so formulated are selected as needed for administration of the inhibitor by any suitable mode known to those skilled in the art.

Parenteral administration modes include intravenous (*i.v.*), subcutaneous (*s.c.*), intraperitoneal (*i.p.*), and intramuscular (*i.m.*) routes. Intravenous administration can be used to obtain acute regulation of peak plasma concentrations of the drug as might be needed. Alternatively, the drug can be administered at a desired rate continuously by *i.v.* catheter. Suitable vehicles include sterile, non-pyrogenic aqueous diluents, such as sterile water for injection, sterile-buffered solutions or sterile saline. The resulting composition is administered to the patient prior to and/or during surgery by intravenous injection or infusion.

Improved half-life and targeting of the drug to phagosomes such as neutrophils and macrophage involved in inflammation may be aided by entrapment of the drug in liposomes. It should be possible to improve the selectivity of liposomal targeting by incorporating into the outside of the liposomes ligands that bind to macromolecules specific to target organs/tissues such as the GI tract and lungs. Alternatively, *i.m.* or *s.c.* deposit injection with or without encapsulation of the drug into degradable microspheres (e.g., comprising poly-DL-lactide-co-glycolide) or protective formulations containing collagen can be used to obtain prolonged sustained drug release. For improved convenience of the dosage form it is possible to use an *i.p.* implanted reservoir and septum such as the percuseal system. Improved convenience and patient compliance may also be achieved by use of either injector pens (e.g., the Novo Pin or Q-pen) or needle-free jet injectors (e.g., from Bioject, Mediject or Becton Dickinson). Precisely controlled release can also be achieved using implantable pumps with delivery to the desired site via a cannula. Examples include the subcutaneously implanted osmotic pumps available from ALZA such as the ALZET osmotic pump.

Nasal delivery may be achieved by incorporating the drug into bioadhesive particulate carriers (<200 mm) such as those comprising cellulose, polyacrylate or polycarbophil, in conjunction with suitable absorption

enhancers such as phospholipids or acylcarnitines. Commercially available systems include those developed by Dan Biosys and Scios Nova.

Pulmonary delivery represents a nonparenteral mode of administration of the drug to the circulation. The lower airway epithelia are highly permeable to a wide range of proteins of molecular sizes up to about 20 kDa. Micron-sized dry powders containing the medicament in a suitable carrier such as mannitol, sucrose or lactose may be delivered to the distal alveolar surface using dry powder inhalers such as those of Inhale™, Dura™, Fisons (Spinhaler™), and Glaxo (Rotahaler™), or Astra (Turbohaler™) propellant based metered dose inhalers. Solution formulations with or without liposomes may be delivered using ultrasonic nebulizers.

Oral delivery may be achieved by incorporating the drug into tablets, coated tablets, dragées, hard and soft gelatin capsules, solutions, emulsions, suspensions or enteric coated capsules designed to release the drug into the colon where digestive protease activity is low. Examples of the latter include the OROS-CT/Osmet™ system of ALZA, and the PULSINCAP™ system of Scherer Drug Delivery Systems. Other systems use azo-crosslinked polymers that are degraded by colon-specific bacterial azoreductases, or pH sensitive polyacrylate polymers that are activated by the rise in pH in the colon. The above systems may be used in conjunction with a wide range of available absorption enhancers. Rectal delivery may be achieved by incorporating the drug into suppositories.

In its preferred medicinal application, for reduction of perioperative blood loss, the preferred mode of administration of the placental bikunin variants of the present invention is parenterally, preferably by *i.v.* route through a central line.

The amount of the pharmaceutical composition to be employed will depend on the recipient and the condition being treated. The requisite amount may be determined without undue experimentation by protocols known to those skilled in the art. Alternatively, the requisite amount may be calculated, based on a determination of the amount of target protease such as plasmin or kallikrein which must be inhibited in order to treat the condition. As the active materials contemplated in this invention are deemed to be nontoxic, treatment preferably involves administration of an excess of the optimally required amount of active agent.

Additionally, placental bikunin, isolated domains or other variants may be used to isolate natural substances such as its cognate proteases from human

material using affinity based separation methods, as well as to elicit antibodies to the protease that can be further used to explore the tissue distribution and useful functions of Placental bikunin.

Searching Human Sequence Data

The existence of a distinct human protein homologous in function to aprotinin, was deduced following a unique analysis of sequence entries to the expressed-sequence-tag data-base (hereafter termed dbEST) at the NCBI (National Center for Biological Information, Maryland). Using the TBlastN algorithm (BLAST, or Basic Local Alignment Search Tool uses the method of Altschul et a., (1990) J. Mol Biol 215: 403-410, to search for similarities between a query sequence and all the sequences in a data-base, protein or nucleic acid in any combination), the data-base was examined for nucleotide sequences bearing homology to the sequence of bovine pre-pro-aprotinin, Trasylol®. This search of numerous clones was selectively narrowed to two particular clones which could possibly encode for a deduced amino acid sequence that would correspond to a human protein homologous in function to aprotinin. The selected nucleic acid sequences were R35464 (SEQ ID NO: 12) and R74593 (SEQ ID NO: 14) that were generated from a human placental nucleic acid library. The translated protein sequence in the longest open reading frame for R35464 (SEQ ID NO: 13) was missing one of the 6 cysteines that are critical for formation of the Kunitz-domain covalent structure, meaning that the nucleic acid sequence of R35464 could not yield a functional inhibitor. Similarly, the longest translated open reading frame from clone R74593 (SEQ ID NO: 15) contained a stop codon 5' to the region encoding the Kunitz like sequence, meaning that this sequence, could not be translated to yield a functional secreted Kunitz domain. The significance of these sequences alone was unclear. It was possible that they represented a) the products of pseudogenes, b) regions of untranslated mRNA, or c) the products of viable mRNA which had been sequenced incorrectly.

Discovery of Human Bikunin

To specifically isolate and determine the actual human sequence, cDNA primers were designed to be capable of hybridizing to sequences located 5' and 3' to the segment of cDNA encoding our proposed Kunitz like sequences found within R35464 and R74593. The primers used to amplify a fragment encoding the Kunitz like sequence of R74593 were:

CGAAGCTTCATCTCCGAAGCTCCAGACG (the 3'primer with a HindIII site; SEQ ID NO:33) and AGGATCTAGACAATAATTACCTGACCAAGGA (the 5'primer with an XbaI site; SEQ ID NO:34).

These primers were used to amplify by PCR (30 cycles) a 500 base pair product from a human placental cDNA library from Clontech (MATCHMAKER, Cat #HL4003AB, Clontech Laboratories, Palo Alto, CA), which was subcloned into Bluescript-SK+ and sequenced with the T3 primer with a Sequenase™ kit version 2.0. Surprisingly, the sequence of the fragment obtained using our primers was different from the sequence listed in the dbEST data base for clone R74593. In particular, our new sequence contained an additional guanosine base inserted 3' to the putative stop codon, but 5' to the segment encoding the Kunitz-like sequence (Figure 3). The insertion of an additional G shifted the stop codon out of the reading frame for the Kunitz-like domain (G at base pair 114 of the corrected sequence for R74593; Figure 3).

Subsequent query of the dbEST for sequences homologous to the Kunitz-like peptide sequence of R74593 yielded H94519 derived from human retina library and N39798. These sequences contained a Kunitz-like sequence that was almost identical to the Kunitz-like domain encoded in R35464 except that it contained all six of the characteristic cysteines. Overlay of each of the nucleotide sequences with that of R74593 (corrected by the insertion of G at b,p, 114) and R35464 was used to obtain a consensus nucleotide sequence for a partial human placental bikunin (SEQ ID NO: 9; Figure 3). The translated consensus sequence yielded an open reading frame extending from residue -18 to +179 (Figure 3; full translation SEQ ID NO: 10) that contained two complete Kunitz-like domain sequences, within the region of amino acid residues 17-64 and 102-159 respectively.

Further efforts attempted to obtain additional 5' sequence by querying dbEST with the sequence of R35464. Possible matches from such searches, that possessed additional 5' sequence were then in turn used to re-query the dbEST. In such an iterative fashion, a series of overlapping 5' sequences were identified which included clones H16866, T66058, R34808, R87894, N40851 and N39876 (Figure 4). Alignment of some of these sequences suggested the presence of a 5' ATG which might serve as a start site for synthesis of the consensus translated protein sequence. From this selected information, it was now possible to selectively screen for, and determine the nucleic acid and polypeptide sequences of a human protein with homologous function to aprotinin.

Re-interrogation of the dbEST revealed a number of new EST entries

shown schematically in Figure 4B. Overlap with these additional ESTs allowed us to construct a much longer consensus oligonucleotide sequence (Figure 4C) that extended both 5' and 3' beyond the original oligonucleotide sequence depicted in Figure 3. In fact, the new sequence of total length 1.6 kilobases extended all the way to the 3' poly-A tail. The increased number of overlapping ESTs at each base-pair position along the sequence improved the level of confidence in certain regions such as the sequence overlapping with the 3' end of EST R74593 (Figure 3). Several overlapping ESTs in this region corroborated two critical base deletions relative to R74593 (located as bold underlined in Figure 4C, map positions 994 and 1005). Translation of the new consensus sequence (Figure 4D) in the bikunin encoding frame yielded a form of placental bikunin that was larger (248 amino acids) than the mature sequence (179 amino acids) encoded from the original consensus (SEQ ID NO: 1), and was terminated by an in-frame stop codon within the oligonucleotide consensus. The size increase was due to a frame shift in the 3' coding region resulting from removal of the two base insertions unique to EST R74593. The frame shift moved the stop codon of the original consensus (Figure 3) out of frame enabling read through into a new frame encoding the additional amino acid sequence. The new translation product (Figure 4D) was identical to the original protein consensus sequence (SEQ ID NO: 1) between residues +1 to +175 (encoding the Kunitz domains), but contained a new C-terminal extension exhibiting a putative 24 residue long transmembrane domain (underlined in Figure 4D) followed by a short 31 residue cytoplasmic domain. The precise sequence around the initiator methionine and signal peptide was somewhat tentative due to considerable heterogeneity amongst the overlapping ESTs in this region.

Analysis of the protein sequence by Geneworks™, highlighted asparagine residues at positions 30 and 67 as consensus sites for putative N-linked glycosylation. Asparagine 30 was not observed during N-terminal sequencing of the full length protein isolated from human placenta, consistent with it being glycosylated.

Cloning of Human Bikunin

The existence of a human mRNA corresponding to the putative human bikunin nucleotide sequence inferred from the analysis of Figure 3, was confirmed as follows. The nucleic acid primer hybridizing 5' to the Kunitz-encoding cDNA sequence of R35464 (b.p. 3-27 of consensus nucleotide

sequence in Figure 3):

GGTCTAGAGGCCGGGTCGTTTCTCGCCTGGCTGGGA

(a 5′ primer derived from R35464 sequence with an XbaI site; SEQ ID NO: 35), and the nucleic acid primer hybridizing 3′ to the Kunitz encoding sequence of R74593 (b.p. 680-700 of consensus nucleotide sequence in Figure 3), was used to PCR amplify, from a Clontech human placental library, a fragment of the size (ca. 670 b.p) expected from a cDNA consensus nucleotide sequence encoding the placental bikunin sequence of Figure 3 (Shown schematically in Figure 4A).

Using a 5′ primer hybridizing to a sequence in R87894 that is 126 b.p 5′ to the putative ATG start site discussed above, (shown schematically in Figure 4A at b.p. 110) plus the same 3′ primer to R74593 as used above, it was possible to amplify a fragment from a Clontech human placental library of the expected size (approximately 872 b.p) predicted by EST overlay (Shown schematically in Figure 4).

Sequencing of the 872 b.p. fragment showed it to contain nucleotide segment corresponding to b.p. 110 to 218 of EST R87894 at its 5′ end and b.p. 310 to 542 of the consensus sequence for placental bikunin inferred from the EST overlay analysis (of Figure 3), at its 3′ end. This 3′ nucleotide sequence contained all of the Kunitz-like domain encoded by placental bikunin (102-159).

To obtain a cDNA encoding the entire extracellular region of the protein, the following 5' PCR primer:

CACCTGATCGCGAGACCCC (SEQ ID NO: 36)

designed to hybridize to a sequence within EST R34808 was used with the same 3′ primer to EST 74593 to amplify (30 cycles) an approximately 780 base-pair cDNA product from the human placental cDNA library. This product was gel purified, and cloned into the TA vector (Invitrogen) for DNA sequencing by the dideoxy method (Sanger F., et al., (1977) Proc. Natl. Acad. Sci (USA), 74: 5463-5467) with the following primers:

Vector Specific:	GATTTAGGTGACACTATAG (SP6) (SEQ ID NO: 37)
	TAATACGACTCACTATAGGG (T7) (SEQ ID NO: 38)
Gene Specific:	TTACCTGACCAAGGAGGAGTGC (SEQ ID NO: 39)
	AATCCGCTGCATTCCTGCTGGTG (SEQ ID NO: 40)
	CAGTCACTGGGCCTTGCCGT (SEQ ID NO: 41)

The resulting cDNA sequence is depicted in Figure 4E together with its

translation product. At the nucleotide level, the sequence exhibited only minor differences from the consensus EST sequence (Figure 4D). Translation of the sequence yielded a coding sequence containing an in-frame initiator ATG site, signal peptide and mature placental bikunin sequence and transmembrane domain. The translated sequence of the PCR product was missing the last 12 amino acid residues from the cytoplasmic domain as a consequence of the choice of selection of the 3' primer for PCR amplification. This choice of 3' PCR primer (designed based on the sequence of R74593) was also responsible for the introduction of an artifactual S to F mutation at amino acid position 211 of the translated PCR-derived sequence. The signal peptide deduced from translation of the PCR fragment was somewhat different to that of the EST consensus.

To obtain a full length placental bikunin cDNA, the PCR derived product (Figure 4E) was gel purified and used to isolate a non-PCR based full length clone representing the bikunin sequence. The PCR derived cDNA sequence was labeled with ^{32}P-CTP by High Prime (Boehringer Mannheim) and used to probe a placental cDNA Library (Stratagene, Unizap™ λ library) using colony hybridization techniques. Approximately 2 X 10^6 phage plaques underwent 3 rounds of screening and plaque purification. Two clones were deemed full length (~1.5 kilobases) as determined by restriction enzyme analysis and based on comparison with the size of the EST consensus sequence (see above). Sequencing of one of these clone by the dideoxy method yielded the oligonucleotide sequence depicted in Figure 4F. The translation product from this sequence yielded a protein with inframe initiator methionine, signal peptide and mature placental bikunin sequence. The mature placental bikunin sequence was identical to the sequence of the mature protein derived by translation of the EST consensus although the signal peptide sequence lengths and sequences differed. Unlike the PCR derived product, the cDNA derived by colony hybridization contained the entire ectodomain, transmembrane domain, cytoplasmic domain and in-frame stop codon. In fact, the clone extended all the way to the poly-A tail. The initiator methionine was followed by a hydrophobic signal peptide which was identical to the signal peptide encoded in the PCR derived clone. Subsequently we expressed and purified a soluble fragment of placental bikunin, bikunin (1-170), from Sf9 cells (Example 9), and found it to be a functional protease inhibitor (Example 10). Furthermore, we isolated from human placenta a soluble fragment of placental bikunin which was also an active protease inhibitor (Example 7). Both the natural protein and the form of the protein expressed in Sf9 cells are probably glycosylated at the asparagine

residue at position 30 based on the recoveries of PTH-amino acids during N-terminal sequencing (Examples 7 and 9).

Based on the above observations, it seems that full length placental bikunin has the capacity to exist as a transmembrane protein on the surface of cells as well as a soluble protein. Other transmembrane proteins that contain Kunitz domains are known to undergo proteolytic processing to yield mixtures of soluble and membrane associated forms. These include two forms of the Amyloid Precursor Protein termed APP751 (Esch F., et al., (1990) Science, 248: 1122-1124) and APP 770 (Wang R., et al., (1991), J. Biol Chem, 266: 16960-16964).

Contact activation is a process which is activated by exposure of damaged vascular surfaces to components of the coagulation cascade. Angiogenesis is a process that involves local activation of plasmin at endothelial surfaces. The specificity of placental bikunin and its putative capacity to anchor to cell surfaces, suggest that the physiologic functions of transmembranous placental bikunin may include regulation of contact activation and angiogenesis.

The amino acid sequences for placental bikunin (7-64), bikunin (102-159), and full length placental bikunin (Figure 4F) were searched against the PIR (Vers. 46.0) and PatchX (Vers. 46.0) protein databases as well as the GeneSeq (Vers. 20.0) protein database of patented sequences using the Genetics Computer Group program FastA. Using the Genetics Computer Group program TFastA (Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. USA 85: 2444-2448), these same protein sequences were searched versus the six-frame translations of the GenBank (Vers. 92.0 with updates to 1/26/96) and EMBL (modified Vers. 45.0) nucleotide databases as well as the GeneSeq (Vers. 20.0) nucleotide database of patented sequences. The EST and STS subsets of GenBank and EMBL were not included in this set of searches. The best matches resulting from these searches contained sequences which were only about 50% identical over their full length to the 58-amino acid protein sequence derived from our analysis of clones R74593 and R35464.

Isolation of Human Bikunin

As mentioned above, synthetic peptides corresponding to bikunin (7-64) and bikunin (102-159) as determined from the translated consensus sequence for bikunin (Figure 3), could be refolded (Examples 2 and 1, respectively) to yield active kallikrein inhibitor protein (Example 4 and 3, respectively). We exploited this unexpected property to devise a purification scheme to isolate

native placental bikunin from human tissue.

Using a purification scheme which employed kallikrein-sepharose affinity chromatography as a first step, highly purified native potent kallikrein inhibitor was isolated. The isolated native human bikunin had an identical N-terminus (sequenced for 50 amino acid residues) as the sequence predicted by the translation of the consensus nucleic acid sequence (Figure 3) amino acid residues +1 to +50 (Example 7). This confirmed for the first time the existence of a novel native kallikrein inhibitor isolated from human placenta.

Known Kunitz-like domains are listed below. Residues believed to be making contact with target proteases are highlighted as of special interest (bold/underlined). These particular residues are named positions Xaa$^{1-16}$ for specific reference as shown by label Xaa below:

```
     Xaa                                        1 1  111      1         1
      1         2  3 456789                     0 1  234      5         6
 1)  IHDFCLVSKVV GRCRASMPRW WYNVTDGSCQ LFVYGGCDGN SNNYLTKEEC LKKCATV
 2)  YEEYCTANAVT GPCRASFPRW YFDVERNSCN NFIYGGCRGN KNSYRSEEAC MLRCFRQ
 3)  -HSFCAFKADD GPCKAIMKRF FFNIFTRQCE EFIYGGCEGN QNRFESLEEC KKMCTRD
 4)  -PDFCFLEEDP GICRGYITRY FYNNQTKQCE RFKYGGCLGN MNNFETLEEC KNICEDG
 5)  -PSWCLTPADR GLCRANENRF YYNSVIGKCR PFKYSGCGGN ENNFTSKQEC LRACKKG
 6)  -AEICLLPLDY GPCRALLLRY YYRYRTQSCR QFLYGGCEGN ANNFYTWEAC DDACWRI
 7)  -PSFCYSPKDE GLCSANVTRY YFNPRYRTCD AFTYTGCGGN DNNFVSREDC KRACAKA
 8)  -KAVCSQEAMT GPCRAVMPRT TFDLSKGKCV RFITGGCGGN RNNFESEDYC MAVCKAM
 9)  RPDFCLEPPYT GPCKARIIRY FYNAKAGLCQ TFVYGGCRAK RNNFKSAEDC MRTCGGA
10)  ----CQLGYSA GPCMGMTSRY FYNGTSMACE TFQYGGCMGN GNNFVTEKEC LQTC
11)   VAACNLPIVR GPCRAFIQLW AFDAVKGKCV LFPYGGCQGN GNKFYSEKEC REYCGVP
12)  -EVCCSEQAET GPCRAMISRW YFDVTEGKCA PFFYGGCGGN RNNFDTEEYC MAVCGSA
13)  ----CKLPKDE GTCRDFILKW YYDPNTKSCA RFWYGGCGGN ENKFGSQKEC EKVC
14)  -PNVCAFPMEK GPCQTYMTRW FFNFETGECE LFAYGGCGGN SNNFLRKEKC EKFCKFT
```

Where sequence number 1) is Bikunin (7-64) (SEQ ID NO: 4); sequence 2) is Bikunin (102-159) (SEQ ID NO: 6); sequence 3) is Tissue factor pathway inhibitor precursor 1 (SEQ ID NO: 18); sequence 4) is Tissue factor pathway inhibitor precursor 1 (SEQ ID NO: 19); sequence 5) is Tissue factor pathway inhibitor precursor (SEQ ID NO: 20); sequence 6) is Tissue factor pathway inhibitor precursor 2 (SEQ ID NO: 21); sequence 7) is Tissue factor pathway inhibitor precursor 2 (SEQ ID NO: 22); sequence 8) is Amyloid precursor protein homologue (SEQ ID NO: 23); sequence 9) is Aprotinin (SEQ ID NO: 24);

sequence 10) is Inter-α-trypsin inhibitor precursor (SEQ ID NOs: 25); sequence 11) is Inter-α-trypsin inhibitor precursor (SEQ ID NOs: 26); sequence 12) is Amyloid precursor protein (SEQ ID NO: 27); sequence 13) is Collagen α-3(VI) precursor (SEQ ID NO: 28); and squence 14) is HKI-B9 (SEQ ID NO: 29).

It can be seen that Placental Bikunin (7-64) and (102-159) each have the same number (six) and spacing of cysteine residues as is found in members of the Kunitz class of serine protease inhibitors. The precise bonding of cysteine residues to form the three intrachain disulfide bonds is known and invarient for all previously known Kunitz family members (Laskowski, M et al., 1980, Ann. Rev. Biochem. 49:593-626). Based on this known bonding pattern and the fact that the folding of Placental Bikunin (7-64) and (102-159) into active protease inhibitors is accompanied by a mass reduction consistent with the formation of three intrachain disulfide bonds (Examples 2 and 1), it is highly probable that the disulfide bonding within the Kunitz domains of Placental Bikunin occur between cysteine residues: C11 and C61; C20 and C44; C36 and C57; C106 and C156; C115 and C139; C131 and C152. Furthermore, this pattern of disulfide bonding is highly probable in larger forms of Placental Bikunin containing both Kunitz domains since such forms of the protein are also active serine protease inhibitors and because N-terminal sequencing (Example 7) of native Placental Bikunin for 50 cycles yielded a sequence that was silent at positions where the cysteine residues were expected.

The placental bikunin, isolated domains or other variants of the present invention may be produced by standard solid phase peptide synthesis using either t-Boc chemistry as described by Merrifield R.B. and Barany G., in: The peptides, Analysis, Synthesis, Biology, 2, Gross E. et al., Eds. Academic Press (1980) Chapter 1; or using F-moc chemistry as described by Carpino L.A., and Han G.Y., (1970) J. Amer Chem Soc., 92, 5748-5749, and illustrated in Example 2. Alternatively, expression of a DNA encoding the placental bikunin variant may be used to produce recombinant placental bikunin variants.

The invention also relates to DNA constructs that encode the Placental bikunin protein variants of the present invention. These constructs may be prepared by synthetic methods such as those described in Beaucage S.L. and Caruthers M.H., (1981) Tetrahedron Lett, 22, pp1859-1862; Matteucci M.D and Caruthers M.H., (1981), J. Am. Chem. Soc. 103, p 3185; or from genomic or cDNA which may have been obtained by screening genomic or cDNA libraries with cDNA probes designed to hybridize with placental bikunin encoding DNA sequence. Genomic or cDNA sequence can be modified at one or more

sites to obtain cDNA encoding any of the amino acid substitutions or deletions described in this disclosure.

The instant invention also relates to expression vectors containing the DNA constructs encoding the placental bikunin, isolated domains or other variants of the present invention that can be used for the production of recombinant placental bikunin variants. The cDNA should be connected to a suitable promoter sequence which shows transcriptional activity in the host cell of choice, possess a suitable terminator and a poly-adenylation signal. The cDNA encoding the placental bikunin variant can be fused to a 5' signal peptide that will result in the protein encoded by the cDNA to undergo secretion. The signal peptide can be one that is recognized by the host organism. In the case of a mammalian host cell, the signal peptide can also be the natural signal peptide present in full length placental bikunin. The procedures used to prepare such vectors for expression of placental bikunin variants are well known in the art and are for example described in Sambrook et al., Molecular Cloning: A laboratory Manual, Cold Spring Harbor, New York, (1989).

The instant invention also relates to transformed cells containing the DNA constructs encoding the placental bikunin, isolated domains or other variants of the present invention that can be used for the production of recombinant placental bikunin variants. A variety of combinations of expression vector and host organism exist which can be used for the production of the placental bikunin variants. Suitable host cells include baculovirus infected Sf9 insect cells, mammalian cells such as BHK, CHO, Hela and C-127, bacteria such as E. coli, and yeasts such as Saccharomyces cervisiae. Methods for the use of mammalian, insect and microbial expressions systems needed to achieve expression of placental bikunin are well known in the art and are described, for example, in Ausubel F.M et al., Current Protocols in Molecular Biology, John Wiley & Sons (1995), Chapter 16. For fragments of placental bikunin containing a single Kunitz inhibitor domain such as bikunin (7-64) and (102-159), yeast and E. coli expression systems are preferable, with yeast systems being most preferred. Typically, yeast expression would be carried out as described in US patent 5,164,482 for aprotinin variants and adapted in Example 5 of the present specification for placental bikunin (102-159). E.coli expression could be carried out using the methods described in US patent 5,032,573. Use of mammalian and yeast systems are most preferred for the expression of larger placental bikunin variants containing both inhibitor domains such as the variant bikunin (7-159).

DNA encoding variants of placental bikunin that possess amino acid substitution of the natural amino sequence can be prepared for expression of recombinant protein using the methods of Kunkel T.A., (1985) Proc. Natl. Acad. Sci USA 82: 488-492. Briefly, the DNA to be mutagenized is cloned into a single stranded bacteriophage vector such as M13. An oligonucleotide spanning the region to be changed and encoding the substitution is hybridized to the single stranded DNA and made double stranded by standard molecular biology techniques. This DNA is then transformed into an appropriate bacterial host and verified by dideoxynucleotide sequencing. The correct DNA is then cloned into the expression plasmid. Alternatively, the target DNA may be mutagenized by standard PCR techniques, sequenced, and inserted into the appropriate expression plasmid.

The following particular examples are offered by way of illustration, and not limitation, of certain aspects and preferred embodiments of the instant invention.

Example 1

Preparation of synthetic placental bikunin (102-159)

Materials and methods/Reagents used. The fluorogenic substrate Tos-Gly-Pro-Lys-AMC was purchased from Bachem BioScience Inc (King of Prussia, PA). PNGB, Pro-Phe-Arg-AMC, Ala-Ala-Pro-Met-AMC, bovine trypsin (type III), human plasma kallikrein, and human plasmin were from Sigma (St. Louis, MO).

Recombinant aprotinin (Trasylol®) was from Bayer AG (Wuppertal, Germany). Pre-loaded Gln Wang resin was from Novabiochem (La Jolla, CA). Thioanisole, ethanedithiol and t-butyl methyl ether was from Aldrich (Milwaukee, WI).

Quantification of functional placental bikunin (7-64) and (102-159)

The amount of trypsin inhibitory activity present in the refolded sample at various stages of purification was measured using GPK-AMC as a substrate. Bovine trypsin (200 pmoles) was incubated for 5 min at 37%C with bikunin (7-64) or (102-159), from various stages of purification, in buffer A (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2 mM $CaCl_2$ and 0.01% triton X-100). GPK-AMC was added (20 μM final) and the amount of coumarin produced was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter over a 2 min. period. For samples being tested the % inhibition

for each was calculated according to equation 1; where R_o is the rate of fluorescence increase in the presence of inhibitor and R_1 is the rate determined in the absence of added sample. One unit of activity for the inhibitor is defined as the amount needed to achieve 50% inhibition in the assay using the conditions as described.

$$\% \text{ inhibition} = 100 \times [1 - R_o/R_1] \qquad (1)$$

Synthesis. Placental bikunin (102-159) was synthesized on an Applied Biosystems model 420A peptide synthesizer using NMP-HBTU Fmoc chemistry. The peptide was synthesized on pre loaded Gln resin with an 8-fold excess of amino acid for each coupling. Cleavage and deprotection was performed in 84.6% trifluoroacetic acid (TFA), 4.4% thioanisole, 2.2% ethanedithiol, 4.4% liquified phenol, and 4.4% H_2O for 2 hours at room temperature. The crude peptide was precipitated, centrifuged and washed twice in t-butyl methyl ether. The peptide was purified on a Dynamax 60A C18 reverse-phase HPLC column using a TFA/acetonitrile gradient. The final preparation (61.0 mg) yielded the correct amino acid composition and molecular mass by Electrospray mass spectroscopy (MH+ =6836.1; calcd = 6835.5) for the predicted sequence:

YEEYCTANAV TGPCRASFPR WYFDVERNSC NNFIYGGCRG NKNSYRSEEA CMLRCFRQ (SEQ ID NO: 6)

Purification. Refolding of placental bikunin (102-159) was performed according to the method of Tam et al., (J. Am. Chem. Soc. 1991, 113: 6657-62). A portion of the purified peptide (15.2 mg) was dissolved in 4.0 ml of 0.1 M Tris, pH 6.0, and 8 M urea. Oxidation of the disulfides was accomplished by dropwise addition of a solution containing 23% DMSO, and 0.1 M Tris, pH 6.0 to obtain a final concentration of 0.5 mg/ml peptide in 20% DMSO, 0.1 M Tris, pH 6.0, and 1 M urea. The solution was allowed to stir for 24 hr at 25°C after which it was diluted 1:10 in buffer containing 50 mM Tris, pH 8.0, and 0.1 M NaCl. The material was purified using a kallikrein affinity column made by covalently attaching 30 mg of bovine pancreatic kallikrein (Bayer AG) to 3.5 mls of CNBr activated Sepharose (Pharmacia) according to the manufacturers instructions. The refolded material was loaded onto the affinity column at a flow rate of 1 ml/min and washed with 50 mM Tris, pH 8.0, and 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The

column was eluted with 3 volumes each of 0.2 M acetic acid, pH 4.0 and 1.7. Active fractions were pooled (see below) and the pH of the solution adjusted to 2.5. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 22.5% acetonitrile in 0.1% TFA. Separation was achieved using a linear gradient of 22.5 to 40% acetonitrile in 0.1% TFA at 1.0 ml/min over 40 min. Active fractions were pooled, lyophilized, redissolved in 0.1% TFA, and stored at -20°C until needed.

Results. Synthetic placental bikunin (102-159) was refolded using 20% DMSO as the oxidizing agent as described above, and purified by a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 1 below).

Table 1

Purification table for the isolation of synthetic placental bikunin (102-159)

TABLE 1

Purification Step	Vol (ml)	mg/ml	mg	Units[c] (U)	SpA (U/mg)	Yield
8.0 M Urea	4.0	3.75[a]	15.0	0	0	-
20% DMSO	32.0	0.47[a]	15.0	16,162	1,078	100
Kallikrein affinity	9.8	0.009[b]	0.09	15,700	170,000	97
C18	3.0	0.013[ab]	0.04	11,964	300,000	74

[a]Protein determined by AAA.
[b]Protein determined by OD280 nm using the extinction coefficient determined for the purified protein (1.7×10^4 $Lmol^{-1}$ cm^{-1}).
[c]One Unit is defined as the amount of material required to inhibit 50% of trypsin activity in a standard assay.

Chromatography of the crude refolded material over an immobilized bovine pancreatic kallikrein column selectively isolated 6.0% of the protein and 97% of the trypsin inhibitory activity present. Subsequent chromatography using C18 reverse-phase yielded a further purification of 2-fold, with an overall recovery of 74%. On RPHPLC, the reduced and refolded placental bikunin (102-159), exhibited elution times of 26.3 and 20.1 minutes, respectively. Mass spectroscopy analysis of the purified material revealed a molecular mass of 6829.8; a loss of 6 mass units from the starting material. This demonstrates the complete formation of the 3 disulfides predicted from the peptide sequence.

The isoelectric points of the purified, refolded synthetic placental bikunin (102-159) was determined using a Multiphor II Electrophoresis System (Pharmacia) run according to the manufacturers suggestions, together with pI standards, using a precast Ampholine® PAGplate (pH 3.5 to 9.5) and focused for 1.5 hrs. After staining, the migration distance from the cathodic edge of the gel to the different protein bands was measured. The pI of each unknown was determined by using a standard curve generated by a plot of the migration distance of standards versus the corresponding pI's. With this technique, the pI of placental bikunin (102-159) was determined to be 8.3, in agreement with the value predicted from the amino acid sequence. This is lower than the value of 10.5 established for the pI of aprotinin. (Tenstad et al., 1994, Acta Physiol. Scand. 152: 33-50).

Example 2

Preparation of synthetic placental bikunin (7-64)

Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications: during refolding, the synthetic peptide was stirred for 30 hr as a solution in 20% DMSO at 25°C; purification by C18 RP-HPLC was achieved with a linear gradient of 25 to 45% acetonitrile in 0.1% TFA over 40 min (1ml/min). Active fractions from the first C18 run were reapplied to the column and fractionated with a linear gradient (60 min, 1 ml/min) of 20 to 40% acetonitrile in 0.1% TFA.

Results. The final purified reduced peptide exhibited an MH+ = 6563, consistent with the sequence:

IHDFCLVSKV VGRCRASMPR WWYNVTDGSC QLFVYGGCDG NSNNYLTKEE CLKKCATV (SEQ ID NO: 4)

The refolding and purification yielded a functional Kunitz domain that was active as an inhibitor of trypsin (Table 2 below).

Table 2A

Purification table for the isolation of synthetic placental bikunin (7-64)

TABLE 2A						
Purification Step	Vol (ml)	mg/ml	mg	Units (U)	SpA (U/mg)	Yield
8.0 M Urea	8.0	2.5	20.0	0	0	-
20% DMSO	64.0	0.31	20.0	68,699	3,435	100
Kall affinity pH 4.0	11.7	0.10	1.16	43,333	36,110	62
Kall affinity pH 1.7	9.0	0.64	5.8	4972	857	7.2
C18-1	4.6	0.14	0.06	21,905	350,143	31.9
C18-2	1.0	0.08	0.02	7,937	466,882	11.5

The purified refolded protein exhibited an MH+ = 6558, i.e. 5±1 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of at least one appropriate disulfide bond.

The pI of placental bikunin (7-64) was determined using the methods employed to determine the pI of placental bikunin (102-159). Placental bikunin (7-64) exhibited a pI that was much higher than the predicted value (pI = 7.9). Refolded placental bikunin (7-64) migrated to the cathodic edge of the gel (pH 9.5) and an accurate pI could not be determined under these conditions.

Continued Preparation of synthetic placental bikunin (7-64)

Because the synthetic placental bikunin (7-64) may not have undergone complete deprotection prior to purification and refolding, refolding was repeated using protein which was certain to be completely deprotected. Placental bikunin (7-64) was synthesized, refolded and purified essentially as described for placental bikunin (102-159) but with the following modifications: during refolding, the synthetic peptide (0.27 mg/ml) was stirred for 30 hr as a solution in 20% DMSO at 25 C; purification by C18 RP-HPLC was achieved with a linear gradient of 22.5 to 50% acetonitrile in 0.1% TFA over 40 min (1 ml/min).

Results. The final purified reduced peptide exhibited an MH+ = 6567.5 , consistent with the sequence:

IHDFCLVSKV VGRCRASMPRW WYNVTDGSC QLFVYGGCDG NSNNYLTKEE CLKKCATV (SEQ ID NO: 4)

The refolding and purification yielded a functional Kunitz domain that was as active as an inhibitor of trypsin (Table 2B below).

Table 2B
Purification table for the isolation of synthetic placental bikunin (7-64)

TABLE 2B

Purification Step	Vol (ml)	mg/ml	mg	Units (U)	SpA (U/mg)	Yield
8.0 M Urea	4.9	2.1	10.5	0	0	-
20% DMSO	39.0	0.27	10.5	236,000	22,500	100
Kallikrein Affinity (pH 2)	14.5	0.3	0.43	120,000	279,070	50.9
C18 Reverse-Phase	0.2	1.2	0.24	70,676	294,483	30.0

The purified refolded protein exhibited an MH+ = 6561.2, i.e. 6.3 mass units less than for the reduced peptide. This demonstrates that refolding caused the formation of the expected three disulfide bonds.

The pI of refolded placental bikunin (7-64) was determined using the methods employed to determine the pI of placental bikunin (102-159). Refolded placental bikunin (7-64) exhibited a pI of 8.85, slightly higher than the predicted value (pI = 7.9).

Example 3

In vitro specificity of functional placental bikunin fragment (102-159)

Proteases. Bovine trypsin, human plasmin, and bovine pancreatic kallikrein quantitation was carried out by active site titration using p-nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase,T., and Shaw, E., (1970) Methods Enzmol., 19: 20-27). Human kallikrein was quantitated by active site titration using bovine aprotinin as a standard and PFR-AMC as a substrate assuming a 1:1 complex formation. The K_m for GPK-AMC with trypsin and plasmin under the conditions used for each enzyme was 29 μM and 726 μM, respectively; the K_m for PFR-AMC with human plasma kallikrein and bovine pancreatic kallikrein was 457 μM and 81.5 μM, respectively; the K_m for AAPR-AMC with elastase was 1600 μM. Human tissue kallikrein (Bayer, Germany) quantification was carried out by active site titration using p'nitrophenyl p'-guanidinobenzoate HCl as previously described (Chase, T., and Shaw, E., (1970) Methods Enzmol. 19: 20-27).

Inhibition Kinetics: The inhibition of trypsin by placental bikunin (102-159) or aprotinin was measured by the incubation of 50 pM trypsin with

placental bikunin (102-159) (0-2 nM) or aprotinin (0-3 nM) in buffer A in a total volume of 1.0 ml. After 5 min. at 37°C, 15 µl of 2 mM GPK-AMC was added and the change in fluorescence (as above) was monitored. The inhibition of human plasmin by placental bikunin (102-159) and aprotinin was determined with plasmin (50 pM) and placental bikunin (102-159) (0-10 nM) or aprotinin (0-4 nM) in buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.02% triton x-100. After 5 min. incubation at 37°C, 25 µl of 20 mM GPK-AMC was added and the change in fluorescence monitored. The inhibition of human plasma kallikrein by placental bikunin (102-159) or aprotinin was determined using kallikrein (2.5 nM) and placental bikunin (102-159) (0-3 nM) or aprotinin (0-45 nM) in 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.02% triton x-100. After 5 min. at 37°C 15 µl of 20 mM PFR-AMC was added and the change in fluorescence monitored. The inhibition of bovine pancreatic kallikrein by placental bikunin (102-159) and aprotinin was determined in a similar manner with kallikrein (92 pM), placental bikunin (102-159) (0-1.6 nM) and aprotinin (0-14 pM) and a final substrate concentration of 100 µM. The apparent inhibition constant K_i^* was determined using the nonlinear regression data analysis program Enzfitter software (Biosoft, Cambridge, UK): The kinetic data from each experiment were analyzed in terms of the equation for a tight binding inhibitor:

$$V_i/V_o = 1 - (E_o + I_o + K_i^* - [(E_o + I_o + K_i^*)^2 - 4\, E_o I_o]^{1/2})/2E_o \qquad (2)$$

where V_i/V_o is the fractional enzyme activity (inhibited vs. uninhibited rate), and E_o and I_o are the total concentrations of enzyme and inhibitor, respectively. Ki values were obtained by correcting for the effect of substrate according to the equation:

$$K_i = K_i^*/(1 + [S_o]/K_m) \qquad (3)$$

(Boudier, C., and Bieth, J. G., (1989) Biochim Biophys Acta., 995: 36-41)

For the inhibition of human neutrophil elastase by placental bikunin (102-159) and aprotinin, elastase (19 nM) was incubated with placental bikunin (102-159) (150 nM) or aprotinin (0-7.5 µM) in buffer containing 0.1 M Tris-HCl (pH 8.0), and 0.05% triton X-100. After 5 min at 37%C, AAPM-AMC (500 µM or 1000 µM) was added and the fluorescence measured over a two-minute period. Ki values were determined from Dixon plots of the form 1/V versus [I]

performed at two different substrate concentrations (Dixon et al., 1979).

The inhibition of human tissue kallikrein by aprotinin, placental bikunin fragment (7-64) or placental bikunin fragment (102-159) was measured by the incubation of 0.35 nM human tissue kallikrein with placental bikunin (7-64) (0-40 nM) or placental bikunin (102-159) (0-2.5 nM), or aprotinin (0-0.5 nM) in a 1 ml reaction volume containing 50 mM Tris-HCl buffer pH 9.0, 50 mM NaCl, and 0.1% triton x-100. After 5 min. at 37°C, 5 ul of 2 mM PFR-AMC was added achieving 10 uM final and the change in fluorescence monitored. The Km for PFR-AMC with human tissue kallikrein under the conditions employed was 5.7 uM. The inhibition of human factor Xa (American Diagnostica, Inc, Greenwich, CT) by synthetic placental bikunin (102-159), recombinant placental bikunin, and aprotinin was measured by the incubation of 0.87 nM human factor Xa with increasing amounts of inhibitor in buffer containing 20 mM Tris (pH 7.5), 0.1 M NaCl, and 0.1% BSA. After 5 min. at 37°C, 30 ul of 20 mM LGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of human urokinase (Sigma) by Kunitz inhibitors was measured by the incubation of urokinase (2.7 ng) with inhibitor in a total volume of 1 ml buffer containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, and 0.1% Triton x-100. After 5 min. at 37°C, 35 ul of 20 mM GGR-AMC (Sigma) was added and the change in fluorescence monitored. The inhibition of Factor XIa (from Enzyme Research Labs, Southbend, IN) was measured by incubating FXIa (0.1 nM) with either 0 to 800 nM placental bikunin (7-64), 0 to 140 nM placental bikunin (102-159) or 0 to 40 uM aprotinin in buffer containing 50 mM Hepes pH 7.5, 100 mM NaCl, 2 mM CaCl2, 0.01% triton x-100, and 1% BSA in a total volume of 1 ml. After 5 min at 37 C, 10 ul of 40 mM Boc-Glu(OBzl)-Ala-Arg-AMC (Bachem Biosciences, King of Prussia, PA) was added and the change in fluorescence monitored.

Results: A direct comparison of the inhibition profiles of placental bikunin (102-159) and aprotinin was made by measuring their inhibition constants with various proteases under identical conditions. The K_i values are listed in Table 3 below.

Table 3
Ki values for the inhibition of various proteases by bikunin (102-159)

TABLE 3

Protease (concentration)	bikunin (102-159) Ki (nM)	Aprotinin Ki (nM)	Substrate (concentration)	Km (mM)
Trypsin (48.5 pM)	0.4	0.8	GPK-AMC (0.03 mM)	0.022
Chymotrypsin (5 nM)	0.24	0.86	AAPF-pNA (0.08 mM)	0.027
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	PFR-AMC (0.1 mM)	0.08
Human Plasma Kallikrein (2.5 nM)	0.3	19.0	PFR-AMC (0.3 mM)	0.46
Human Plasmin (50 pM)	1.8	1.3	GPK-AMC (0.5 mM)	0.73
Human Neutrophil Elastase (19 nM)	323.0	8500.0	AAPM-AMC (1.0 µM)	1.6
Factor XIIa	>300.0	12,000.0	PFR-AMC (0.2 µM)	0.35
Human Tissue Kallikrein (0.35 nM)	0.13	0.004	PFR-AMC (10 µM)	0.0057
factor Xa (0.87 nM)	274	N.I. at 3 µM	LGR-AMC (0.6 mM)	N.D.
urokinase	11000	4500	GGR-AMC (0.7 mM)	N.D.
factor XIa (0.1 nM)	15	288	E(OBz)AR-AMC (0.4 mM)	0.46

Placental bikunin (102-159) and aprotinin inhibit bovine trypsin and human plasmin to a comparable extent under the conditions employed. Aprotinin inhibited elastase with a Ki of 8.5 µM. Placental bikunin (102-159) inhibited elastase with a Ki of 323nM. The K_i value for the placental bikunin (102-159) inhibition of bovine pancreatic kallikrein was 20-fold higher than that of aprotinin inhibition. In contrast, placental bikunin (102-159) is a more potent inhibitor of human plasma kallikrein than aprotinin and binds with a 56-fold higher affinity.

Because placental bikunin (102-159) is greater than 50 times more potent than Trasylol® as an inhibitor of kallikrein, smaller amounts of human placental bikunin, or fragments thereof (i.e. placental bikunin (102-159)) are needed than Trasylol® in order to maintain the effective patient doses of inhibitor in KIU. This reduces the cost per dose of the drug and reduces the likelihood of adverse nephrotoxic effects upon re-exposure of the medicament to patients. Furthermore, the protein is human derived, and thus much less immunogenic in man than aprotinin which is derived from cows. This results in significant reductions in the risk of incurring adverse immunologic events upon re-exposure of the medicament to patients.

Example 4

In vitro specificity of functional placental bikunin fragment (7-64)

In vitro specificity of functional human placental bikunin (7-64) was determined using the materials and methods as described in the Examples above.

Results: The table below shows the efficacy of placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

Table 4 A

Ki values for the inhibition of various proteases by bikunin(7-64)

TABLE 4A

Protease (concentration)	bikunin(7-64) Ki (nM)	Aprotinin Ki (nM)	bikunin (102-159) Ki (nM)
Trypsin (48.5 pM)	0.17	0.8	0.4
Bovine Pancreatic Kallikrein (92.0 pM)	0.4	0.02	0.4
Human Plasma Kallikrein (2.5 nM)	2.4	19.0	0.3
Human Plasmin (50 pM)	3.1	1.3	1.8
Bovine chymotrypsin (5 nM)	0.6	0.9	0.2
Factor XIIa	>300	12000	>300
elastase	>100	8500	323

The results show that the amino acid sequence encoding placental bikunin (7-64) can be refolded to obtain an active serine protease inhibitor that is effective against at least four trypsin-like serine proteases.

Table 4B below also shows the efficacy of refolded placental bikunin (7-64) as an inhibitor of various serine proteases *in vitro*. Refolded placental bikunin (7-64) was prepared from protein that was certain to be completely deprotected prior to purification and refolding. Data is shown compared against data obtained for screening inhibition using either placental bikunin (102-159), or aprotinin (Trasylol®).

Table 4B
Ki values for the inhibition of various proteases by refolded bikunin (7-64)

TABLE 4B			
Protease (concentration)	bikunin (7-64) Ki (nM)	Aprotinin Ki (nM)	bikunin (102-159) Ki (nM)
Trypsin (50 pM)	0.2	0.8	0.3
Human Plasma Kallikrein (0.2 nM)	0.7	19.0	0.7
Human Plasmin (50 pM)	3.7	1.3	1.8
Factor XIIa	not done	12,000	4,500
Factor XIa (0.1 nM)	200	288	15
Human Tissue Kallikrein	2.3	0.004	0.13

Suprisingly, placental bikunin (7-64) was more potent than aprotinin at inhibiting human plasma kallikrein, and at least similar in efficacy as a plasmin inhibitor. These data show that placental bikunin (7-64) is at least as effective as aprotinin, using *in vitro* assays, and that one would expect better or similar potency *in vivo*.

Example 5

Expression of placental bikunin variant (102-159) in yeast

The DNA sequence encoding placental bikunin 102-159 (SEQ ID NO: 6) was generated using synthetic oligonucleotides. The final DNA product consisted (5' to 3') of 15 nucleotides from the yeast α-mating factor propeptide sequence fused to the in-frame cDNA sequence encoding placental bikunin (102-159), followed by an in-frame stop codon. Upon cloning into a yeast expression vector pS604, the cDNA would direct the expression of a fusion protein comprising an N-terminal yeast α-mating factor propeptide fused to the 58 amino acid sequence of placental bikunin (102-159). Processing of this fusion protein at a KEX-2 cleavage site at the junction between the α-mating factor and Kunitz domain was designed to liberate the Kunitz domain at its native N-terminus.

A 5' sense oligonucleotide of the following sequence and containing a HindIII site for cloning was synthesized:

```
GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC
GCC AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC
TGG TAC TTT GAC GTG GAG AGG (SEQ ID NO: 42)
```

A 3' antisense oligonucleotide of the following sequence and containing both a BamHI site for cloning and a stop codon was synthesized:

```
CGC GGA TCC CTA CTG GCG GAA GCA GCG GAG CAT GCA GGC CTC
CTC AGA GCG GTA GCT GTT CTT ATT GCC CCG GCA GCC TCC ATA
GAT GAA GTT ATT GCA GGA GTT CCT CTC CAC GTC AAA GTA CCA
GCG
(SEQ ID NO: 43)
```

The oligonucleotides were dissolved in 10 mM Tris buffer pH 8.0 containing 1 mM EDTA, and 12 ug of each oligo were added combined and brought to 0.25M NaCl. To hybridize, the oligonucleotides were denatured by boiling for 5 minutes and allowed to cool from 65°C to room temp over 2 hrs. Overlaps were extended using the Klenow fragment and digested with HindIII and BamHI. The resulting digested double stranded fragment was cloned into pUC19 and sequence confirmed. A clone containing the fragment of the correct sequence was digested with BamHI/HindIII to liberate the bikunin containing fragment with the following + strand sequence:

```
GAA GGG GTA AGC TTG GAT AAA AGA TAT GAA GAA TAC TGC ACC
GCC AAC GCA GTC ACT GGG CCT TGC CGT GCA TCC TTC CCA CGC
TGG TAC TTT GAC GTG GAG AGG AAC TCC TGC AAT AAC TTC ATC
TAT GGA GGC TGC CGG GGC AAT AAG AAC AGC TAC CGC TCT GAG
GAG GCC TGC ATG CTC CGC TGC TTC CGC CAG TAG GGA TCC (SEQ
ID.: 44)
```

which was then gel purified and ligated into BamHI/HindIII cut pS604. The ligation mixture was extracted into phenol/chloroform and purified over a S-200 minispin column. The ligation product was directed transformed into yeast strains SC101 and WHL341 and plated on ura selection plates. Twelve colonies from each strain were re-streaked on ura drop out plates. A single colony was inoculated into 2 ml of ura DO media and grown over night at 30°C. Cells were pelleted for 2 minutes at 14000x g and the supernatants evaluated for their content of placental bikunin (102-159).

Detection of expression of placental bikunin (102-159) in transformed yeast

Firstly, the supernatants (50 ul per assay) were evaluated for their capacity to inhibit the *in vitro* activity of trypsin using the assay methods as described in Example 1 (1 ml assay volume). An un-used media only sample as well as a yeast clone expressing an inactive variant of aprotinin served as

negative controls. A yeast clone expressing natural aprotinin served as a positive control and is shown for comparison.

The second method to quantify placental bikunin (102-159) expression exploited use of polyclonal antibodies (pAbs) against the synthetic peptide to monitor the accumulation of the recombinant peptide using Western blots. These studies were performed only with recombinants derived from strain SC101, since these produced greater inhibitory activity than recombinants derived from strain WHL341.

To produce the pAb, two 6-8 week old New Zealand White female rabbits (Hazelton Research Labs, Denver, Pa) were immunized on day zero with 250 ug of purified reduced synthetic placental bikunin (102-159), in Complete Freund's adjuvant, followed by boosts on days 14, 35 and 56 and 77 each with 125 ug of the same antigen in Incomplete Freund's adjuvant. Antiserum used in the present studies was collected after the third boost by established procedures. Polyclonal antibodies were purified from the antiserum over protein A.

Colonies 2.4 and 2.5 from transformation of yeast SC101 (Figure 8) as well as an aprotinin control were grown overnight in 50 ml of ura DO media at 30°C. Cells were pelleted and the supernatant concentrated 100-fold using a Centriprep 3 (Amicon, Beverly, MA) concentrator. Samples of each (30 μl) were subjected to SDS-PAGE on 10-20% tricine buffered gels (Novex, San Diego, CA) using the manufacturers procedures. Duplicate gels were either developed with a silver stain kit (Integrated Separation Systems, Nantick, MA) or transferred to nitrocellulose and developed with the purified polyclonal antibody elicited to synthetic bikunin (102-159). Alkaline-phosphatase conjugated goat anti-rabbit antibody was used as the secondary antibody according to the manufacturer's directions (Kirkegaard and Perry, Gaithersburg, MD).

Purification of placental bikunin (102-159) from a transformed strain of SC101

Fermentation broth from a 1L culture of SC101 strain 2.4 was harvested by centrifugation (4,000 g x 30 min.) then applied to a 1.0 ml column of anhydrochymotrypsin-sepharose (Takara Biochemical Inc., CA), that was previously equilibrated with 50 mM Hepes buffer pH 7.5 containing 0.1M NaCl, 2 mM $CaCl_2$ and 0.01% (v/v) triton X-100. The column was washed with the same buffer but containing 1.0 M NaCl until the A280nm declined to zero, whereupon the column was eluted with 0.1M formic acid pH 2.5. Eluted fractions were pooled and applied to a C18 column (Vydac, 5um, 4.6 x 250 mm)

previously equilibrated with 0.1% TFA, and eluted with a 50 min. linear gradient of 20 to 80% acetonitrile in 0.1% TFA. Fractions containing placental bikunin (102-159) were pooled and re-chromatographed on C18 employing elution with a linear 22.5 to 50% acetonitrile gradient in 0.1% TFA.

Results. Figure 8 shows the percent trypsin activity inhibited by twelve colonies derived from the transformation of each of strains SC101 and WHL341. The results show that all twelve colonies of yeast strain SC101 transformed with the trypsin inhibitor placental bikunin (102-159) had the ability to produce a substantial amount of trypsin inhibitory activity compared to the negative controls both of which showed no ability to inhibit trypsin. The activity is therefore related to the expression of a specific inhibitor in the placental bikunin variant (102-159) transformed cells. The yeast WHL341 samples contained minimal trypsin inhibitory activity. This may be correlated to the slow growth observed with this strain under the conditions employed.

Figure 9 shows the SDS-PAGE and western analysis of the yeast SC101 supernatants. Silver stained SDS-PAGE of supernatants derived from recombinant yeasts 2.4 and 2.5 expressing placental bikunin (102-159) as well as from the yeast expressing aprotinin yielded a protein band running at approximated 6 kDa, corresponding to the size expected for each recombinant Kunitz inhibitor domain. Western analysis showed that the 6 kDa bands expressed by stains 2.4. and 2.5 reacted with the pAb elicited to placental bikunin (102-159). The same 6 kDa band in the aprotinin control did not react with the same antibody, demonstrating the specificity of the antibody for the placental bikunin variant (102-159).

The final preparation of placental bikunin C-terminal domain was highly pure by silver-stained SDS-PAGE (Figure 10). The overall recovery of broth-derived trypsin inhibitory activity in the final preparation was 31%. N-terminal sequencing of the purified inhibitor indicated that 40% of the protein is correctly processed to yield the correct N-terminus for placental bikunin (102-159) while about 60 % of the material contained a portion of the yeast α-mating factor. The purified material comprised an active serine protease inhibitor exhibiting an apparent Ki of 0.35 nM for the *in vitro* inhibition of plasma kallikrein.

In conclusion, the accumulation both of a protease inhibitor activity and a protein immunochemically related to synthetic bikunin (102-159) in fermentation broth as well as the isolation of placental bikunin (102-159) from

one of the transformed lines provided proof of expression of placental bikunin in the recombinant yeast strains described herein, showing for the first time the utility of yeasts for the production of placental bikunin fragments.

Additional constructs were prepared in an effort to augment the expression level of the Kunitz domain contained within placental bikunin 102-159, as well as to increase the yield of protein with the correct N-terminus. We hypothesized that the N-terminal residues of placental bikunin 102-159 (YEEY--) may have presented a cleavage site that is only poorly recognized by the yeast KEX-2 protease that enzymically removes the yeast a-factor pro-region. Therefore, we prepared yeast expression constructs for the production of placental bikunin 103-159 (N-terminus of EEY...), 101-159 (N-terminus of NYEEY...) and 98-159 (DMFNYEEY..) in order to modify the P' subsites surrounding the KEX-2 cleavage site. To attempt to augment the levels of recombinant protein expression, we also used the yeast preferred codons rather than mammalian preferred codons in preparing some of the constructs described below. The constructs were essentially prepared as described above for placental bikunin 102-159 (defined as construct #1) but with the following modifications:

Construct #2 placental bikunin 103-159, yeast codon usage

A 5' sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAGAAGAA TACTGTACTG CTAATGCTGT TACTGGTCCA TGTAGAGCTT CTTTTCCAAG ATGGTACTTT GATGTTGAAA GA (SEQ ID NO: 55)

and 3' antisense oligonucleotide

ACTGGATCCT CATTGGCGAA AACATCTCAA CATACAGGCT TCTTCAGATC TGTAAGAATT TTTATTACCT CTACAACCAC CGTAAATAAA ATTATTACAA GAATTTCTTT CAACATCAAA GTACCATCT (SEQ ID NO: 56)

were manipulated as described for the production of an expression construct (construct #1 above) for the expression of placental bikunin 102-159

Construct #3 placental bikunin 101-159, yeast codon usage

A 5′ sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAAATTAC GAAGAATACT GTACTGCTAA TGCTGTTACT GGTCCATGTA GAGCTTCTTT TCCAAGATGG TACTTTGATG TTGAAAGA (SEQ ID NO: 57)

and the same 3′ antisense oligonucleotide as used for construct #2, were manipulated as described for the production of an expression construct (construct #1 above) for the expression of placental bikunin 102-159.

Construct #4 placental bikunin 98-159, yeast codon usage

A 5′ sense oligonucleotide

GAAGGGGTAA GCTTGGATAA AAGAGATATG TTTAATTACG AAGAATACTG TACTGCTAAT GCTGTTACTG GTCCATGTAG AGCTTCTTTT CCAAGATGGT ACTTTGATGT TGAAAGA (SEQ ID NO: 58)

and the same 3′ antisense oligonucleotide as used for construct #2, were manipulated as described for the production of an expression construct (construct #1 above).

Yeast strain SC101 (MATα, ura 3-52, suc 2) was transformed with the plasmids containing each of the above cDNAs, and proteins were expressed using the methods that were described above for the production of placental bikunin 102-159 with human codon usage. Approximately 250 ml of each yeast culture was harvested, and the supernatant from centrifugation (15 min x 3000 RPM) separately subjected to purification over 1 ml columns of kallikrein-sepharose as described above. The relative amount of trypsin inhibitory activity in the applysate, the amount of purified protein recovered and the N-terminal sequence of the purified protein were determined and are listed below in Table 7.

Table 7
Relative production levels of different proteins containing the C-terminal Kunitz domain of placental bikunin

TABLE 7

Construct		Relative conc. of inhibitor in applysate	N-terminal sequencing: amount (pmol)	sequence	Comments
#2	103-159	none detected	none	none	no expression
#3	101-159	25 % inhibition	none	none	low expression
#4 expression correct product	98-159	93 % inhibition	910	DMFNYE-	good
#1	102-159	82 % inhibition	480	AKEEGV-	expression of active incorrectly processed protein

The results show that placental bikunin fragments of different lengths that contain the C-terminal Kunitz domain show wide variation in capacity to express functional secreted protein. Constructs expressing fragments 101-159 and 103-159 yielded little or low enzymic activity in the supernatants prior to purification, and N-terminal sequencing of 0.05 ml aliquots of each purified fraction yielded undetectable amounts of inhibitor. On the other hand expression either of placental bikunin 102-159 or 98-159 yielded significant amounts of protease activity prior to purification. N-terminal sequencing however showed that the purified protein recovered from expression of 102-159 was once again largely incorrectly processed, exhibiting an N-terminus consistent with processing of the majority of the pre-protein at a site within the yeast α-mating factor pro-sequence. The purified protein recovered from expression of placental bikunin 98-159 however was processed entirely at the correct site to yield the correct N-terminus. Furthermore, nearly twice as much protein was recovered as compared to the recovery of placental bikunin 102-159. Placental bikunin 98-159 thus represents a preferred fragment length for the production of the C-terminal Kunitz domain of placental bikunin by the α-mating factor pre-pro sequence/ KEX-2 processing system of *S. cerevisiae*,

Example 6

Alternative procedure for yeast expression

The 58 amino acid peptide derived from the R74593 translation product can also be PCR amplified from either the R87894-R74593 PCR product cloned into the TA vector™ (Invitrogen, San Diego, CA) after DNA sequencing or from human placental cDNA. The amplified DNA product will consist of 19 nucleotides from the yeast α-mating factor leader sequence mated to the R74593 sequence which codes for the YEEY--CFRQ (58 residues) so as to make the translation product in frame, constructing an α -mating factor/Kunitz domain fusion protein. The protein sequence also contains a kex 2 cleavage which will liberate the Kunitz domain at its native N-terminus.

The 5' sense oligonucleotide which contains a HindIII site for cloning will contain the following sequence:

GCCAAGCTTG GATAAAAGAT ATGAAGAAT ACTGCACCGC CAACGCA
(SEQ ID NO: 30)

The 3' antisense oligonucleotide contains a BamHI site for cloning as well as a stop codon and is of the following sequence:

GGGGATCCTC ACTGCTGGCG GAAGCAGCGG AGCAT (SEQ ID NO: 31)

The full 206 nucleotide cDNA sequence to be cloned into the yeast expression vector is of the following sequence:

CCAAGCTTGG ATAAAAGATA TGAAGAATAC TGCACCGCCA ACGCAGTCAC
TGGGCCTTGC CGTGCATCCT TCCCACGCTG GTACTTTGAC GTGGAGAGGA
ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC
TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGTGAGG
ATCCCC (SEQ ID NO: 32)

After PCR amplification, this DNA will be digested with HindIII, BamHI and cloned into the yeast expression vector pMT15 (see US patent 5,164,482, incorporated by reference in the entirety) also digested with HindIII and BamHI. The resulting plasmid vector is used to transform yeast strain SC 106 using the methods described in US patent 5,164,482. The URA 3+ yeast transformants are isolated and cultivated under inducing conditions. The yield of recombinant Placental bikunin variants is determined according to the

amount of trypsin inhibitory activity that accumulated in the culture supernatants over time using the *in vitro* assay method described above. Fermentation broths are centrifuged at 9000 rpm for 30 minutes. The supernatant is then filtered through a 0.4 then a 0.2 μm filter, diluted to a conductivity of 7.5 ms, and adjusted to pH 3 with citric acid. The sample is then batch absorbed onto 200 ml of S-sepharose fast flow (Pharmacia) in 50 mM sodium citrate pH 3 and stirred for 60 min. The gel is subsequently washed sequentially with 2 L of each of: 50 mM sodium citrate pH 3.0; 50 mM Tris-HCL pH 9.0; 20 mM HEPES pH 6.0. The washed gel is transferred into a suitable column and eluted with a linear gradient of 0 to 1 M sodium chloride in 20 mM HEPES pH 6.0. Eluted fractions containing *in vitro* trypsin inhibitory activity are then pooled and further purified either by a) chromatography over a column of immobilized anhydrotrypsin (essentially as described in Example 2); b) by chromatography over a column of immobilized bovine kallikrein; or c) a combination of conventional chromatographic steps including gel filtration and/or anion-exchange chromatography.

Example 7
Isolation and characterization of native human placental bikunin from placenta

Bikunin protein was purified to apparent homogeniety from whole frozen placenta (Analytical Biological Services, Inc, Wilmington, DE). The placenta (740 gm) was thawed to room temperature and cut into 0.5 to 1.0 cm pieces, placed on ice and washed with 600 ml PBS buffer. The wash was decanted and 240 ml of placenta pieces placed into a Waring blender. After adding 300 ml of buffer consisting of 0.1 M Tris (pH 8.0), and 0.1 M NaCl , the mixture was blended on high speed for 2 min, decanted into 750.0 ml centrifuge tubes, and placed on ice. This procedure was repeated until all material was processed. The combined slurry was centrifuged at 4500 x g for 60 minutes at 4°C. The supernatant was filtered through cheese cloth and the placental bikunin purified using a kallikrein affinity column made by covalently attaching 70 mg of bovine pancreatic kallikrein (Bayer AG) to 5.0 mls of CNBr activated Sepharose (Pharmacia) according to manufacturers instruction. The material was loaded onto the affinity column at a flow rate of 2.0 ml/min and washed with 0.1 M Tris (pH 8.0), 0.1 M NaCl until absorbance at 280 nm of the wash could no longer be detected. The column was further washed with 0.1 M Tris (pH 8.0), 0.5 M NaCl and then eluted with 3 volumes of 0.2 M acetic acid,

pH 4.0. Fractions containing kallikrein and trypsin inhibitory (see below) activity were pooled, frozen, and lyophilized. Placental bikunin was further purified by gel-filtration chromatography using a Superdex 75 10/30 (Pharmacia) column attached to a Beckman System Gold HPLC system. Briefly, the column was equilibrated in 0.1 M Tris, 0.15 M NaCl, and 0.1% Triton X-100 at a flow rate of 0.5 ml/min. The lyophilized sample was reconstituted in 1.0 ml of 0.1 M Tris, pH 8.0 and injected onto the gel-filtration column in 200 µl aliquots. Fractions were collected (0.5 ml) and assayed for trypsin and kallikrein inhibitory activity. Active fractions were pooled, and the pH of the solution adjusted to 2.5 by addition of TFA. The material was directly applied to a Vydac C18 reverse-phase column (5 micron, 0.46 x 25 cm) which had been equilibrated in 20% acetonitrile in 0.1 %TFA. Separation was achieved using a linear gradient of 20 to 80% acetonitrile in 0.1% TFA at 1.0 ml/min over 50 minutes after an initial 20 minute wash at 20% acetonitrile in 0.1% TFA. Fractions (1ml) were collected and assayed for trypsin and kallikrein inhibitory activity. Fractions containing inhibitory activity were concentrated using a speed-vac concentrator (Savant) and subjected to N-terminal sequence analysis.

Functional assays for Placental Bikunin:

Identification of functional placental bikunin was achieved by measuring its ability to inhibit bovine trypsin and human plasma kallikrein. Trypsin inhibitory activity was performed in assay buffer (50 mM Hepes, pH 7.5, 0.1 M NaCl, 2.0 mM CaCl2, 0.1% Triton x-100) at room temperature in a 96-well microtiter plate (Perkin Elmer) using Gly-Pro-Lys-Aminomethylcoumarin as a substrate. The amount of coumarin produced by trypsin was determined by measuring the fluorescence (ex = 370 nm, em = 432 nm) on a Perkin-Elmer LS-50B fluorimeter equipped with a plate reader. Trypsin (23 µg in 100 µl buffer) was mixed with 20 µl of the sample to be tested and incubated for 10 minutes at 25°C. The reaction was started by the addition of 50 µl of the substrate GPK-AMC (33 µM final) in assay buffer. The fluorescence intensity was measured and the % inhibition for each fraction was determined by:

$$\% \text{ inhibition} = 100 \times [1 - Fo/F1]$$

where Fo is the fluorescence of the unknown and F1 is the fluorescence of the trypsin only control. Kallikrein inhibitory activity of the fractions was similarly measured using 7.0 nM kallikrein in assay buffer (50 mM Tris, pH 8.0, 50 mM

NaCl, 0.1% triton x-100) and 66.0 μM Pro-Phe-Arg-AMC as a substrate.

Determination of the *in vitro* specificity of placental bikunin

The *In vitro* specificity of native human placental bikunin was determined using the materials and methods as described in the preceding examples above. Placental bikunin was quantified by active site titration against a known concentration of trypsin using GPK-AMC as a substrate to monitor the fraction of unbound trypsin.

Protein Sequencing

The 1 ml fraction (C18-29 Delaria) was reduced to 300 ml in volume, on a Speed Vac, to reduce the amount of organic solvent. The sample was then loaded onto a Hewlett-Packard miniature biphasic reaction column, and washed with 1 ml of 2% trifluoroacetic acid. The sample was sequenced on a Hewlett-Packard Model G1005A protein sequencing system using Edman degradation. Version 3.0 sequencing methods and all reagents were supplied by Hewlett-Packard. Sequence was confirmed for 50 cycles.

Results. Placental Bikunin was purified to apparent homogeniety by sequential kallikrein affinity, gel-filtration, and reverse-phase chromatography (see purification table below):

Table 5
Purification table for native Placental Bikunin (1-179)

TABLE 5

Step	Vol (ml)	OD 280 (/ml)	OD 280	Units[a] (U)	Units/OD 280
Placenta Supernatant	1800.0	41.7	75,060	3,000,000	40.0
Kallikrein Affinity pH 4.0	20.0	0.17	3.36	16,000	4,880
Kallikrein Affinity pH 1.7	10.2	0.45	4.56	12,000	2,630
Superdex 75	15.0	0.0085	0.13	3,191	24,546

[a]One Unit is defined as that amount which inhibits 50% of trypsin activity in a standard assay.

The majority of the kallikrein and trypsin inhibitory activity eluted from the kallikrein affinity column in the pH 4.0 elution. Subsequent gel-filtration chromatography (Figure 5) yielded a peak of kallikrein and trypsin inhibitory

activity with a molecular weight range of 10 to 40 kDa as judged by a standard curve generated by running molecular weight standards under identical conditions. Reverse-phase C18 chromatography (Figure 6) yielded 4 peaks of inhibitory activity with the most potent eluting at approximately 30 % acetonitrile. The activity associated with the first peak to elute from C18 (fraction 29) exhibited an amino acid sequence starting with amino acid 1 of the predicted amino acid sequence of placental bikunin (ADRER...; SEQ ID NO: 1), and was identical to the predicted sequence for 50 cycles of sequencing (underlined amino acids in Figure 3). Cysteine residues within this sequence stretch were silent as expected for sequencing of oxidized protein. The cysteine residues at amino acid positions 11 and 20 of mature placental bikunin were later identified from sequencing of the S-pyridylethylated protein whereupon PTH-pyridylethyl-cysteine was recovered at cycles 11 and 20.

Interestingly, the asparagine at amino acid residue number 30 of the sequence (Figure 3) was silent showing that this site is likely to be glycosylated. Fraction 29 yielded one major sequence corresponding to that of placental bikunin starting at residue #1 (27 pmol at cycle 1) plus a minor sequence (2 pmol) also derived from placental bikunin starting at residue 6 (SIHD...). This shows that the final preparation sequenced in fraction 29 is highly pure, and most likely responsible for the protease inhibitory activity associated with this fraction (Figure 6).

Accordingly, the final preparation of placental bikunin from C18 chromatography was highly pure based on a silver-stained SDS-PAGE analysis (Figure 7), where the protein migrated with an apparent Mr of 24 kDa on a 10 to 20 % acrylamide tricine gel (Novex, San Diego, CA) calibrated with the following molecular weight markers: insulin (2.9 kDa); bovine trypsin inhibitor (5.8 kDa); lysozyme (14.7 kDa); β-lactaglobulin (18.4 kDa); carbonic anhydrase (29 kDa); and ovalbumin (43 kDa). The above size of placental bikunin on SDS-PAGE is consistent with that predicted from the full length coding sequence (Figure 4F).

As expected based on the N-terminal sequencing results described above, the purified protein reacted with an antibody elicited to placental bikunin (7-64) to yield a band with the same Mr (Figure 12A) as observed for the purified preparation detected on gels by silver stain (Figure 7). However, when the same preparation was reacted with an antibody elicited to synthetic placental bikunin (102-159), a band corresponding to the full length protein was not observed. Rather, a fragment that co-migrated with synthetic bikunin (102-

159) of approximately 6 kDa was observed. The simplest interpretation of these results is that the purified preparation had undergone degradation subsequent to purification to yield an N-terminal fragment comprising the N-terminal domain and a C-terminal fragment comprising the C-terminal domain. Assuming that the fragment reactive against antiserum to placental bikunin (7-64) is devoid of the C-terminal end of the full length protein, the size (24 kDa) would suggest a high state of glycosylation.

Table 6. below shows the potency of *in vitro* inhibition of various serine proteases by placental bikunin. Data are compared with that obtained with aprotinin (Trasylol®).

Table 6
Ki values for the inhibition of various proteases by placental bikunin

TABLE 6

Protease (concentration)	Placental Bikunin Ki (nM)	Aprotinin Ki (nM)
Trypsin (48.5 pM)	0.13	0.8
Human Plasmin (50 pM)	1.9	1.3

The results show that placental bikunin isolated from a natural source (human placenta) is a potent inhibitor of trypsin-like serine proteases.

Example 8

Expression pattern of placental bikunin amongst different human organs and tissues

A multiple tissue northern was purchased from Clontech which contained 2 μg of polyA+ RNA from human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. Two different cDNA probes were used: 1) a gel purified cDNA encoding placental bikunin (102-159); 2) the 780 base pair PCR-derived cDNA (Figure 4E) liberated from a TA clone by digestion with EcoRI and gel purified. Each probe was labeled using ^{32}P-dCTP and a random priming labeling kit from Boehringer Mannheim Biochemicals (Indiana), then used to hybridize to the multiple tissue northern according to the manufacturers specifications. Autoradiographs were generated using Biomax film with an 18 hr exposure time, and developed using a Umax Scanner and scanned using Adobe Photoshop.

Results. The pattern of tissue expression observed using a placental bikunin (102-159) probe (Figure 11A) or a larger probe containing both Kunitz domains of placental bikunin (Figure 11B) was essentially the same as might be expected. The placental bikunin mRNA was most abundant in pancreas and placenta. Significant levels were also observed in lung, brain and kidney, while lower levels were observed in heart and liver, and the mRNA was undetectable in skeletal muscle. The transcript size was 1.95 kilobases in all cases, in close agreement with the predicted size of placental bikunin deduced both from EST overlay and cloning of full length cDNA described in preceding sections.

The broad tissue distribution of the mRNA shows that placental bikunin is broadly expressed. Since the protein also contains a leader sequence it would have ample exposure to the human immune system, requiring that it become recognized as a self protein. Additional evidence for a broad tissue distribution of placental bikunin mRNA expression was derived from the fact that some of the EST entries with homology to placental bikunin (Figure 4B) were derived from human adult and infant brain, and human retina, breast, ovary, olfactory epithelium, and placenta. It is concluded therefore that administration of the native human protein to human patients would be unlikely to elicit an immune response.

Interestingly, the expression pattern of placental bikunin is somewhat reminiscent of that for bovine aprotinin which is found in high levels in bovine lung and pancreas. To further elucidate the expression pattern of placental bikunin, RT-PCR of total RNA from the following human cells was determined: un-stimulated human umbilical vein endothelial cells (HUVECs), HK-2 (line derived from kidney proximal tubule), TF-1 (erythroleukemia line) and phorbolester (PMA)-stimulated human peripheral blood leukocytes. The probes used:

CACCTGATCGCGAGACCCC (sense; SEQ ID NO: 59);
CTGGCGGAAGCAGCGGAGCATGC (antisense; SEQ ID NO: 60),

were designed to amplify a 600 b.p placental bikunin encoding cDNA fragment. Comparisons were normalized by inclusion of actin primers to amplify an 800 b.p. actin fragment. Whereas the 800 b.p fragment identified on agarose gels with ethidium bromide was of equal intensity in all lanes, the 600 b.p. placental bikunin fragment was absent from the HUVECs but present in significant amounts in each of the other cell lines. We conclude that placental

bikunin is not expressed in at least some endothelial cells but is expressed in some leukocyte populations.

Example 9

Purification and properties of Placental Bikunin (1-170) highly purified from a Baculovirus / Sf9 expression system

A large fragment of Placental bikunin containing both Kunitz domains (Placental Bikunin 1-170) was expressed in Sf9 cells as follows. Placental bikunin cDNA obtained by PCR (Figure 4E) and contained within a TA vector (see previous Examples) was liberated by digestion with HindIII and Xba1 yielding a fragment flanked by a 5' XbaI site and 3' HindIII site. This fragment was gel purified and then cloned into the M13mp19 vector (New England Biolabs, Beverly, MA). In vitro mutagenesis (Kunkel T.A., (1985) Proc. Natl. Acad. Sci. USA, 82: 488-492) was used to generate a Pst1 site 3' to the XbaI site at the 5' end, but 5' to the sequence encoding the ATG start site, natural placental bikunin signal peptide and mature placental bikunin coding sequence. The oligonucleotide used for the mutagenesis had the sequence:

5' CGC GTC TCG GCT GAC CTG GCC CTG CAG ATG GCG CAC GTG TGC GGG 3' (SEQ ID NO: 61)

A stop codon (TAG) and BglII / XmaI site was similarly engineered at the 3' end of the cDNA using the oligonucleotide:

5' CTG CCC CTT GGC TCA AAG TAG GAA GAT CTT CCC CCC GGG GGG GTG GTT CTG GCG GGG CTG 3' (SEQ ID NO: 62).

The stop codon was in frame with the sequence encoding placental bikunin and caused termination immediately following the Lysine at amino acid residue 170, thus encoding a truncated placental bikunin fragment devoid of the putative transmembrane domain. The product from digestion with Pst1 and BglII was isolated and cloned into the BacPac8 vector for expression of Placental bikunin fragment (1-170) which contains both Kunitz domains but which is truncated immediately N-terminal to the putative transmembrane segment.

The expression of Bikunin by Sf-9 insect cells was optimal at a multiplicity of infection of 1 to 1 when the medium was harvested at 72 h post

infection. After harvesting, the baculovirus cell culture supernatant (2L) was adjusted to pH 8.0 by the addition of Tris-HCl. Bikunin was purified by chromatography using a 5 ml bovine pancreatic kallikrein affinity column as previously described in Example 7 for the purification of native placental bikunin from placenta. Eluted material was adjusted to pH 2.5 with TFA and subjected to chromatography on a C18 reverse-phase column (1.0 x 25 cm) equilibrated in 10% acetonitrile in 0.1% TFA at a flow rate of 1 ml/min. The bikunin was eluted with a linear gradient of 10 to 80% acetonitrile in 0.1% TFA over 40 min. Active fractions were pooled, lyophilized, redissolved in 50 mM Hepes (pH 7.5), 0.1 M NaCl, 2 mM $CaCl_2$, and 0.1% triton x-100, and stored at -20°C until needed. The concentration of recombinant bikunin was determined by amino acid analysis.

Results. Recombinant bikunin was purified from baculovirus cell culture supernatant using a 2-step purification protocol as shown below, to yield an active trypsin inhibitor (Table 8 below).

Table 8
Purification of recombinant bikunin from transformed culture supernatant

TABLE 8

Purification Step	Vol (ml)	OD 280/ml	OD 280 total	Units (U)	Specific activity (U/OD)
Supernatant	2300.0	9.0	20,700	6,150,000	297
Kallikrein affinity	23.0	0.12	2.76	40,700	14,746
C18 reverse-phase	0.4	3.84	1.54	11,111	72,150

Chromatography of the crude material over an immobilized bovine pancreatic kallikrein affinity column selectively isolated 0.013 % of the protein and 0.67 % of the trypsin inhibitory activity present. The majority of the trypsin inhibitory activity present in the starting supernatant did not bind to the immobilized kallikrein and is not related to bikunin (results not shown). Subsequent chromatography using C18 reverse-phase yielded a further purification of 5-fold, with a recovery of 0.2%. The final preparation was highly pure by SDS-PAGE (Figure 13), exhibiting an Mr of 21.3 kDa, and reacted on immunoblots to rabbit anti-placental bikunin 102-159 (not shown). N-terminal sequencing (26 cycles) yielded the expected sequence for mature placental bikunin (Figure 4F) starting at residue +1(ADRER....) , showing that the signal

peptide was correctly processed in Sf9 cells.

Purified placental bikunin from Sf9 cells (100 pmol) was pyridylethyl-alkylated, CNBr digested and then sequenced without resolution of the resulting fragments. Sequencing for 20 cycles yielded the following N-terminii:

Sequence	Amount	Placental bikunin residue #
LRCFrQQENPP-PLG-----	21 pmol	154 - 168 (SEQ ID NO: 63)
ADRERSIHDFCLVSKVVGRC	20 pmol	1 - 20 (SEQ ID NO: 64)
FNYeEYCTANAVTGPCRASF	16 pmol	100 - 119 (SEQ ID NO: 65)
Pr--Y-V-dGS-Q-F-Y-G	6 pmol	25 - 43 (SEQ ID NO: 66)

Thus N-terminii corresponding to each of the expected four fragments were recovered. This confirms that the Sf9 expressed protein contained the entire ectodomain sequence of placental bikunin (1-170). N-terminal sequencing (50 cycles) of an additional sample of undigested Placental Bikunin (1-170) resulted in an amino acid sequence which at cycle 30 was devoid of any PTH-amino acid (PTH-asparagine was expected). A similar result was obtained upon sequencing of the natural protein from human placenta (Example 7) and is consistent with this residue being glycosylated as predicted from the amino acid sequence surrounding this asparagine residue. Furthermore, the cysteine residues within this region were also silent consistent with their participation in disulfide bonding.

Example 10

Inhibition specificity of purified placental bikunin derived from Sf9 cells.

The *in vitro* specificity of recombinant bikunin was determined using the materials and methods as described in Examples 3, 4 and 7. In addition, the inhibition of human tissue kallikrein by bikunin was measured by the incubation of 0.35 nM human tissue kallikrein recombinant bikunin in buffer containing 50 mM Tris (pH 9.0), 50 mM NaCl, and 0.01% triton x-100. After 5 min. at 37°C, 5 µl of 2 mM PFR-AMC was added and the change in fluorescence monitored.

Inhibition of tissue plasminogen activator (tPA) was also determined as follows: tPA (single chain form from human melanoma cell culture from Sigma Chemical Co, St Louis, MO) was pre-incubated with inhibitor for 2 hr at room temperature in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl, and 0.02% sodium azide. Reactions were subsequently initiated by transfer to a reaction

system comprising the following initial component concentrations: tPA (7.5 nM), inhibitor 0 to 6.6 μM, DIle-Lpro-Larg-pNitroaniline (1mM) in 28 mM Tris buffer pH 8.5 containing 0.004 % (v/v) triton x-100 and 0.005% (v/v) sodium azide. Formation of p-Nitroaniline was determined from the A405nm measured following incubation at 37 C for 2hr.

The table below show the efficacy of recombinant bikunin as an inhibitor of various serine proteases *in vitro*. Data is shown compared against data obtained for screening inhibition using either recombinant bikunin, or aprotinin.

Table 9
Comparisons of Ki values for the inhibition of various proteases by recombinant placental bikunin (1-170) or aprotinin

TABLE 9

Protease (concentration)	Recombinant Bikunin Ki (nM)	Aprotinin Ki (nM)
Trypsin (48.5 pM)	0.064	0.8
Human Plasma Kallikrein (2.5 nM)	0.18	19.0
Human Tissue Kallikrein (0.35 nM)	0.04	0.004
Bovine Pancreatic Kallikrein (100 pM)	0.12	0.02
Human Plasmin (50 pM)	0.23	1.3
factor Xa (0.87 nM)	180	5% Inhibition at 31 μM
factor XIa (0.1 nM)	3.0	288
tissue plasminogen activator (7.5 nM)	< 60	no inhibition at 6.6 μM
Tissue Factor VIIa	800	no inhibition at 1 μM

The results show that recombinant bikunin can be expressed in insect cells to yield an active protease inhibitor that is effective against at least five different serine protease inhibitors. Recombinant bikunin was more potent than aprotinin against human plasma kallikrein, trypsin and plasmin. Surprisingly, the recombinant bikunin was more potent that the synthetically derived bikunin fragments (7-64) and (102-159) against all enzymes tested. These data show that recombinant bikunin is more effective than aprotinin, using *in vitro* assays, and that one would expect better *in vivo* potency.

Besides measuring the potencies against specific proteases, the capacity of placental bikunin (1-170) to prolong the activated partial thromboplastin time (APTT) was evaluated and compared with the activity associated with

aprotinin. Inhibitor was diluted in 20 mM Tris buffer pH 7.2 containing 150 mM NaCl and 0.02% sodium azide and added (0.1 ml) to a cuvette contained within an MLA Electra[R] 800 Automatic Coagulation Timer coagulometer (Medical Laboratory Automation, Inc., Pleasantville, N.Y.). The instrument was set to APTT mode with a 300 sec. activation time and the duplicate mode. Following addition of 0.1 ml of plasma (Specialty Assayed Reference Plasma lot 1-6-5185, Helena Laboratories, Beaumont, TX), the APTT reagent (Automated APTT-lot 102345, from Organon Teknika Corp., Durhan, NC) and 25 mM CaCl2 were automatically dispensed to initiate clotting, and the clotting time was monitored automatically. The results (Figure 14) showed that a doubling of the clotting time required approximately 2 μM final aprotinin, but only 0.3 μM Sf9 derived placental bikunin. These data show that placental bikunin is an effective anticoagulant, and usefull as a medicament for diseases involving pathologic activation of the intrinsic pathway of coagulation.

Although certain embodiments of the invention have been described in detail for the purpose of illustration, it will be readily apparent to those skilled in the art that the methods and formulations described herein may be modified without departing from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

WE CLAIM:

1. A substantially purified protein, having serine protease inhibitory activity, selected from the group of proteins consisting of materials each of which comprises one of the following amino acid sequences, the amino acids of said sequences being numbered in accordance with the amino acid sequence of native human placental bikunin shown in figure 4F in which the N-terminal residue generated by removal of signal peptide is designated as residue 1:

```
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN    50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF   100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE   150
ACMLRCFRQQ ENPPLPLGSK                                    170
(SEQ ID NO: 52);

MAQLCGL RRSRAFLALL GSLLLSGVLA                             -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN    50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF   100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE   150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN   200
QERALRTVWS SGDDKEQLVK NTYVL                              225
(SEQ ID NO: 49);

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN    50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF   100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE   150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN   200
QERALRTVWS SGDDKEQLVK NTYVL                              225
(SEQ ID NO: 70);

    AGSFLAWL GSLLLSGVLA  -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN    50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF   100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE   150
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS                          179
(SEQ ID NO: 2);
```

MLR AEADGVSRLL GSLLLSGVLA -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS SGDDKEQLVK NTYVL 225
(SEQ ID NO: 45);

MAQLCGL RRSRAFLALL GSLLLSGVLA -1
ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS FGD 213
(SEQ ID NO: 47);

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
QERALRTVWS FGD 213
(SEQ ID NO: 71);

IHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATV 64
(SEQ ID NO: 4);

CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK C 61
(SEQ ID NO: 5);

YEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRCFRQ 159
(SEQ ID NO: 6);

CTANAVTGPC RASFPRWYFD VERNSCNNFI YGGCRGNKNS YRSEE 150
ACMLRC 156

(SEQ ID NO: 7);

IHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPPRQ DSEDHSSDMF 75
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125
ACMLRCFRQ 159
(SEQ ID NO: 3);

CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
ACMLRC 156
(SEQ ID NO: 50);

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 25
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 75
NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 125
ACMLRCFRQQ ENPPLPLGSK VVVLAGAVS 179
(SEQ ID NO: 1); and

ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN 50
YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DS 92
(SEQ ID NO: 8).

2. A protein as in claim 1, wherein said protein is glycosylated, or contains at least one intra-chain cysteine-cysteine disulfide bond, or is both glycosylated and contains at least one intra-chain cysteine-cysteine disulfide bond.

3. A pharmaceutical composition for inhibiting serine protease activity, comprising a protein of claim 1 or claim 2 plus a pharmaceutically acceptable carrier.

4. An isolated nucleic acid sequence which encodes for a protein of claim 1.

5. A self-replicating protein expression vector containing a nucleic acid sequence which encodes for and is capable of expressing a protein of claim or claim 2.

6. A method for inhibiting serine protease activity comprising contacting serine protease with an effective amount of at least one protein of claim 1 or claim 2.

7. A method for treating a condition of brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, polytrauma, stroke, cerebral or subarachnoid hemorrhage, inflamation of the brain, inflamation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, open heart surgery, gastric cancer, cervical cancer, or prevention of metastasis comprising administering to a subject having such a condition an effective amount of the protein of claim 1 or claim 2.

8. The method of Claim 7 wherein said condition is brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, stroke, cerebral or subarachnoid hemorrhage, inflamation of the brain, inflamation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, or open heart surgery.

9. The method of Claim 7 wherein said condition is gastric cancer, cervical cancer, or prevention of metastasis.

10. A method for the preparation of a medicament for the treatment of brain edema, spinal cord edema, multiple sclerosis, ischemia, perioperative blood loss, sepsis, septic shock, fibrosis, disease associated with pathologic blood coagulation or clotting, stroke, cerebral or subarachnoid hemorrhage, inflamation of the brain, inflamation of the spinal cord, cerebral infection, cerebral granulomatosis, spinal infection, spinal granulomatosis, open heart surgery, gastric cancer, cervical cancer, or prevention of metastasis.

11. A method for preparing a protien of claim 1 or claim 2 using recombinant DNA technology.

FIGURE 1

```
R35464 GGCCGGGTCG TTTCTCGCCT GGCTGGGATC GCTGCTCCTC TCTGGGGTCC   50
ORF       P  G  R   F  S  P   G  W  D  R   C  S  S   L  G  S    16

R35464 TGGCCGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  100
ORF    W  P  A  D   R  E  R   S  I  H   D  F  C  L   V  S  K    33

R35464 GGTGGTGGGC AGATTCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA  150
ORF     V  V  G   R  E  R  A   S  M  P   R  W  W   Y  N  V  T   50

R35464 CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC  200
ORF       D  G  S   C  Q  L   F  V  Y  G   G  C  D   G  N  S    66

R35464 AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC  250
ORF    N  N  Y  L   T  K  E   E  C  L   K  K  C  A   T  V  T    83

R35464 AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT  300
ORF     E  N  A   T  G  D  L   A  T  S   R  N  A   A  D  S  S  100

R35464 CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CTTGAAGACC ACTTCAGCGA  350
ORF       V  P  S   A  P  R   R  Q  D  S   *  R  P   L  Q  R   116

R35464 TATGTTTCAA NTATTGNAAG AATAATTGCA CCGNCAACGN ATT-------  393
ORF    Y  V  S  *   I  *  R   I  I  A   P  *  T  *              130
```

KEY
R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO: 12)
ORF = EST R35464 Open Reading Frame Translation (SEQ ID NO: 13)

FIGURE 2

```
R74593 GCAATAATTA CCTGACCAAG GAGGAGTGCC TCAAGAAATG TGCCACTGTC   50
ORF     Q  *  L  P  D  Q  G  G  V  P  Q  E  M  C  H  C  H    17

R74593 ACAGAGAATG CCACGGGTGA CCTGGCCACC AGCAGGAATG CAGCGGATTC  100
ORF      R  E  C  H  G  *  P  G  H  Q  Q  E  C  S  G  F      33

R74593 CTCTGTCCCA AGTCTCCCAG AAGGCAGGAT TCTGAAGACC ACTCCAGCGA  150
ORF    L  C  P  K  S  P  R  R  Q  D  S  E  D  H  S  S  D     50

R74593 TATGTTCAAC TATGAAGAAT ACTGCACCGC CAACGCAGTC ACTGGGCCTT  200
ORF     M  F  N  Y  E  E  Y  C  T  A  N  A  V  T  G  P  C    67

R74593 GCCGTGCATC CTTCCCACGC TGGTACTTTG ACGTGGAGAG GAACTCCTGC  250
ORF      R  A  S  F  P  R  W  Y  F  D  V  E  R  N  S  C      83

R74593 AATAACTTCA TCTATGGAGG CTGCCGGGGC AATAAGAACA GCTACCGCTC  300
ORF    N  N  F  I  Y  G  G  C  R  G  N  K  N  S  Y  R  S    100

R74593 TGAGGAGGCC TGCATGCTCC GCTGCTTCCG CCAGCAGGAG AATCCTCCCC  350
ORF     E  E  A  C  M  L  R  C  F  R  Q  Q  E  N  P  P  L   117

R74593 TGCCCCTTGG CTCAAAGGTG GTGGTTCTGG CCGGGGCTGT TTCGTGATGG  400
ORF      P  L  G  S  K  V  V  V  L  A  G  A  V  S  *  W     133

R74593 TGTTGATCCT TTTCCTGGGG AGCNTCCATG GTCTTACTGA TTCCGGGTGG  450
ORF    C  *  S  F  S  W  G  A  S  M  V  L  L  I  P  G  G    150

R74593 CAAGGAGGAA CCAGGAGCGT GCCCTGCGGA NCGTCTGGAG CTTCGGAGAT  500
ORF     K  E  E  P  G  A  C  P  A  X  R  L  E  L  R  R  *   167

R74593 GACAAGGGNT                                             510
ORF      Q  G                                                169
```

KEY
R74593 = Nucleic acid sequence of EST R74593 (SEQ ID NO: 14)
ORF = EST R74593 Open Reading Frame Translation (SEQ ID NO: 15)

FIGURE 3

```
R35464        GGCCGGGTCGT TTCTCGCCTG GCTGGGA-TC GCTGCTCCTC TCTGGGGTCC  50
N39798                               TGGGANTC GCTGCTCCTC TCTGGGGTCC  28
H94519         GCNGCG-CGT TNNTCGCNT- GCTGGGA-TC GCTGCACCTC TCTGGGGTCG  47
R74593 corr.   ---------- ---------- ---------- ---------- ----------
Consensus     GGCCGGGTCGT TTCTCGCCTG GCTGGGA-TC GCTGCTCCTC TCTGGGGTCC  50
Translation     A  G  S  F  L  A  W  L  G  S  L  L  L  S  G  V       -3

R35464         TGGCCGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA 100
N39798         TGG-CGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  77
H94519         NGG-CGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  96
R74593 corr.   ---------- ---------- ---------- ---------- ----------
Consensus      TGG-CGGCCG ACCGAGAACG CAGCATCCAC GACTTCTGCC TGGTGTCGAA  99
Translation     L  A  A  D  R  E  R  S  I  H  D  E  C  L  V  S  K   15

R35464         GGTGGTGGGC AGATTCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 150
N39798         GGTGGTGGGC AGATGCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 127
H94519         GGTGGTGGGC AGATGCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 146
R74593 corr.   ---------- ---------- ---------- ---------- ----------
Consensus      GGTGGTGGGC AGATGCCGGG CCTCCATGCC TAGGTGGTGG TACAATGTCA 149
Translation     V  V  G  R  C  R  A  S  M  P  R  W  W  Y  N  V  T   32

R35464         CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 200
N39798         CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 177
H94519         CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 196
R74593 corr.   ---------- ---------- ---------- ---------- --------GC   2
Consensus      CTGACGGATC CTGCCAGCTG TTTGTGTATG GGGGCTGTGA CGGAAACAGC 199
Translation     D  G  S  C  Q  L  F  V  Y  G  G  C  D  G  N  S      48

R35464         AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 250
N39798         AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 227
H94519         AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 246
R74593 corr.   AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC  52
Consensus      AATAATTACC TGACCAAGGA GGAGTGCCTC AAGAAATGTG CCACTGTCAC 249
Translation     N  N  Y  L  T  K  E  E  C  L  K  K  C  A  T  V  T   65

R35464         AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 300
N39798         AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 277
H94519         AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 296
R74593 corr.   AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 102
Consensus      AGAGAATGCC ACGGGTGACC TGGCCACCAG CAGGAATGCA GCGGATTCCT 299
Translation     E  N  A  T  G  D  L  A  T  S  R  N  A  A  D  S  S   82

R35464         CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CTTGAAGACC ACTTCAGCGA 350
N39798         CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 326
H94519         CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 345
R74593 corr.   CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 151
Consensus      CTGTCCCAAG TGCTCCCAGA AGGCAGGATT CT-GAAGACC ACTCCAGCGA 348
Translation      V  P  S  A  P  R  R  Q  D  S  E  D  H  S  S  D    98

R35464         TATGTTTCAA NTATTGNAAG AATAATTGCA CCGNCAACGN ATT------- 393
N39798         TATGTT-CAA CTA-TG-AAG AATACT-GCA CCGCCAACGC AGTCACTGGG 372
H94519         TATGTT-CAA CTA-TG-AAG AATACTGGCA CCGCCAACGC ATTCACTGGG 392
R74593 corr.   TATGTT-CAA CTA-TG-AAG AATACT-GCA CCGCCAACGC AGTCACTGGG 197
Consensus      TATGTT-CAA CTA-TG-AAG AATACT-GCA CCGCCAACGC AGTCACTGGG 394
Translation     M  F  N  Y  E  E  Y  C  T  A  N  A  V  T  G         113
```

FIGURE 3 (CON'T)

```
R35464          ---------- ---------- ---------- ---------- ----------
N39798          CCTTGC-GTG GAATCCTTTC CCACGCTGGN AATTTNGACG TTGAGAAGGA 421
H94519          CCT-GC-GTG -CATCCTT-C CCACGCTGGT ACTTT-GNCG ---------- 427
R74593 corr.    CCTTGCCGTG -CATCCTT-C CCACGCTGGT ACTTT-GACG TGGAGA-GGA 243
Consensus       CCTTGCCGTG -CATCCTT-C CCACGCTGGT ACTTT-GACG TGGAGA-GGA 440
Translation     P  C  R  A    S  F    P  R  W  Y    F  D  V   E  R   N 129

R35464          ---------- ---------- ---------- ---------- ----------
N39798          AC-------- ---------- ---------- ---------- ---------- 423
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC 293
Consensus       ACTCCTGCAA TAACTTCATC TATGGAGGCT GCCGGGGCAA TAAGAACAGC 490
Translation      S  C  N   N  F  I    Y  G  G  C   R  G  N   K  N  S   145

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGGAGAA 343
Consensus       TACCGCTCTG AGGAGGCCTG CATGCTCCGC TGCTTCCGCC AGCAGGAGAA 540
Translation     Y  R  S  E   E  A  C   M  L  R   C  F  R  Q   Q  E  N  162

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    TCCTCCCCTG CCCCTTGGCT CAAAGGTGGT GGTTCTGGCC GGGGCTGTTT 393
Consensus       TCCTCCCCTG CCCCTTGGCT CAAAGGTGGT GGTTCTGGCC GGGGCTGTTT 590
Translation      P  P  L   P  L  G  S   K  V  V   V  L  A   G  A  V  S 179

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    CGTGATGGTG TTGATCCTTT TCCTGGGGAG CNTCCATGGT CTTACTGATT 443
Consensus       CGTGATGGTG TTGATCCTTT TCCTGGGGAG CNTCCATGGT CTTACTGATT 640
Translation      *  W  C   *  S  F    S  W  G  A   S  M  V   L  L  I   195

R35464          ---------- ---------- ---------- ---------- ----------
N39798          ---------- ---------- ---------- ---------- ----------
H94519          ---------- ---------- ---------- ---------- ----------
R74593 corr.    CCGGGTGGCA AGGAGGAACC AGGAGCGTGC CCTGCGGANC GTCTGGAGCT 493
Consensus       CCGGGTGGCA AGGAGGAACC AGGAGCGTGC CCTGCGGANC GTCTGGAGCT 690
Translation     P  G  G  K   E  E  P   G  A  C   P  A  *  R   L  E  L  212

R35464          ---------- --------
N39798          ---------- --------
H94519          ---------- --------
R74593 corr.    TCGGAGATGA CAAGGGNT                                    511
Consensus       TCGGAGATGA CAAGGGNT                                    708
Translation      R  R  *   Q  G                                        217
```

KEY
R35464 = Nucleic acid sequence of EST R35464 (SEQ ID NO.: 12)
N39798 = Nucleic acid sequence of EST N39798 (SEQ ID NO.: 17)
H94519 = Nucleic acid sequence of EST H94519 (SEQ ID NO.: 16)
R74593 corr. = Corrected version of (SEQ ID NO.: 14) G at b.p. 114
Consensus = Nucelic acid sequence for human bikunin (SEQ ID NO.: 9)
Translation = Amino acid Translation of Consensus (SEQ ID NO.: 10)

Figure 4 A.

Schematic depicting the overlap of ESTs bearing homology to the cDNA sequence encoding placental bikunin

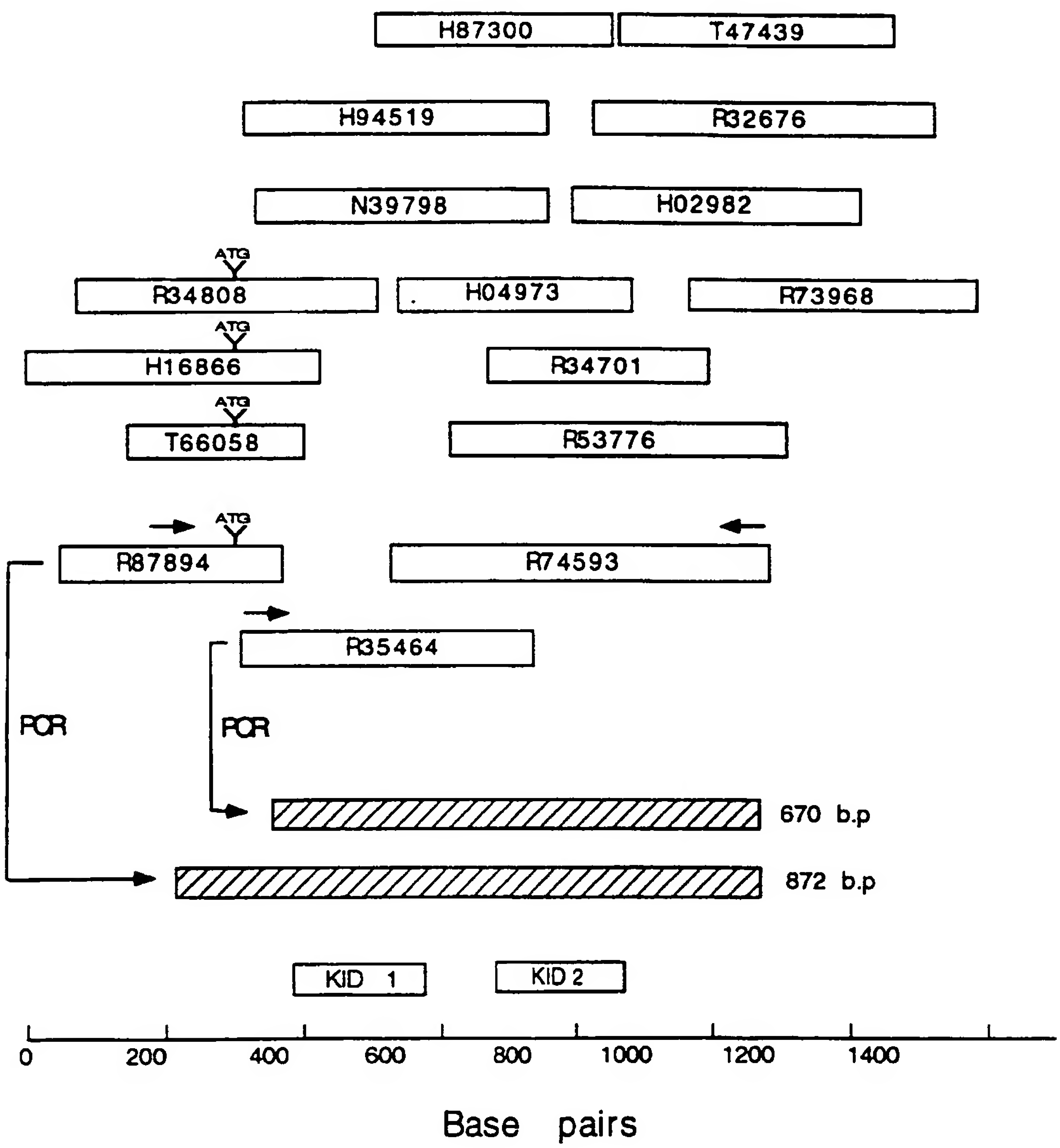
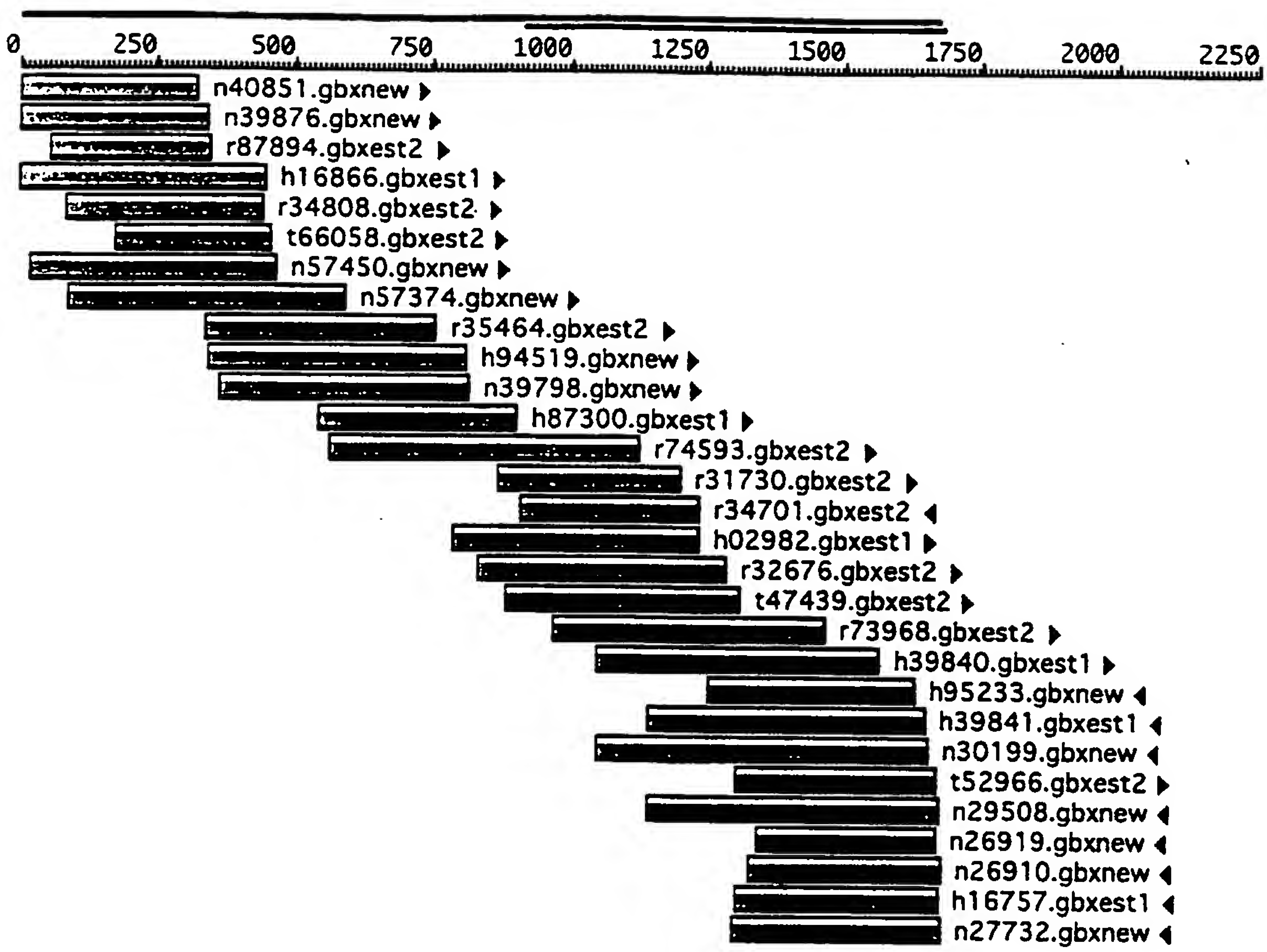


Figure 4B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250
500
750
1000
1250
1500
1750
2000
2250
n40851.gbxnew
n39876.gbxnew
r87894.gbxest2
h16866.gbxest1
r34808.gbxest2
t66058.gbxest2
n57450.gbxnew
n57374.gbxnew
r35464.gbxest2
h94519.gbxnew
n39798.gbxnew
h87300.gbxest1
r74593.gbxest2
r31730.gbxest2
r34701.gbxest2
h02982.gbxest1
r32676.gbxest2
t47439.gbxest2
r73968.gbxest2
h39840.gbxest1
h95233.gbxnew
h39841.gbxest1
n30199.gbxnew
t52966.gbxest2
n29508.gbxnew
n26919.gbxnew
n26910.gbxnew
h16757.gbxest1
n27732.gbxnew

Figure 4C

```
         1                                                  50
Bikunin  ......GCGA CCTCCGCGCG TTGGGAGGTG TAGCGCGGCT CTGAACGCGT
N40851   ......GCGA CCTCCGCGCG TTGGGAGGTG TAGCGCGGCT CTGAACGCGT
N39876   ......GCGA CCTCCGCGCG TTGGGAGGTG TAGCGCGGCT CTGAACGCGT
R87894   .......... .......... .......... .......... ..........
H16866   .....GGCGA CCTCCGCGCG TTGGGAGGTG TAGCGCG.CT CTGAACGGGN
R34808   .......... .......... .......... .......... ..........
T66058   .......... .......... .......... .......... ..........
N57450   .......... .......... .........T TAGCGCGGCT CTGAACGCNA
N57374   .......... .......... .......... .......... ..........
R35464   .......... .......... .......... .......... ..........
H94519   .......... .......... .......... .......... ..........
N39798   .......... .......... .......... .......... ..........
H87300   .......... .......... .......... .......... ..........
R74593   .......... .......... .......... .......... ..........
R31730   .......... .......... .......... .......... ..........
R34701   .......... .......... .......... .......... ..........
H02982   .......... .......... .......... .......... ..........
R32676   .......... .......... .......... .......... ..........
T47439   .......... .......... .......... .......... ..........
R73968   .......... .......... .......... .......... ..........
H39840   .......... .......... .......... .......... ..........
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   .......... .......... .......... .......... ..........
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

	51				100
Bikunin	GNA GGGCCG	TTGAGTGTCG	CAGGCGGCGA	GGGCGCGAGT	GAGGAGCAGA
N40851	NGAGNGGCCG	TTGAGTGTCG	CAGGCGGCGA	GGGCGCGAGT	GAGGAGCAGA
N39876	GCA.GGGCCG	TTGAGTGTCG	CAGGCGGCGA	GGGCGCGAGT	GAGGAGCAGA
R87894	....	TTGAGTGTNG	NAGGCGGCGA	GGGCGCGAGT	GAGGAGCAGA
H16866	.:ANGGGCCG	TTGAGTGTCG	CAGGCGGC.A	GGGCN.GAGT	GAGGAGCAGA
R34808	....	....	....	.........G	GAGGAGCAGA
T66058	....	....	....	....	....
N57450	GAAGNGGCCG	TTGAGTGTCG	CAGGCGGCGA	GGGCGCGAGT	GAGGAGCAGA
N57374	....	....	....	....	.......AGA
R35464	....	....	....	....	....
H94519	....	....	....	....	....
N39798	....	....	....	....	....
H87300	....	....	....	....	....
R74593	....	....	....	....	....
R31730	....	....	....	....	....
R34701	....	....	....	....	....
H02982	....	....	....	....	....
R32676	....	....	....	....	....
T47439	....	....	....	....	....
R73968	....	....	....	....	....
H39840	....	....	....	....	....
H95233	....	....	....	....	....
H39841	....	....	....	....	....
N30199	....	....	....	....	....
T52966	....	....	....	....	....
N29508	....	....	....	....	....
N26919	....	....	....	....	....
N26910	....	....	....	....	....
H16757	....	....	....	....	....
N27732	....	....	....	....	....

Figure 4C (Con't)

```
         101                                                      150
Bikunin  CCCAGGCATC GCGCGCCGAG AAGNC GGGC GTCCCCACAC TGAAGGTCCG
 N40851  CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 N39876  CCCAGGCATC GCGCGCCGAG AAGNC.GGGC NTCCCCACAC TGAAGGTCCG
 R87894  CCCAGGCATC GCGCGCCGAG AAGGCCGGGC GTCCCCACAC TGAAGGTCCG
 H16866  CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 R34808  CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 T66058  .......... .......... .......... .......... ..........
 N57450  CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 N57374  CCCAGGCATC GCGCGCCGAG AAGNC.GGGC GTCCCCACAC TGAAGGTCCG
 R35464  .......... .......... .......... .......... ..........
 H94519  .......... .......... .......... .......... ..........
 N39798  .......... .......... .......... .......... ..........
 H87300  .......... .......... .......... .......... ..........
 R74593  .......... .......... .......... .......... ..........
 R31730  .......... .......... .......... .......... ..........
 R34701  .......... .......... .......... .......... ..........
 H02982  .......... .......... .......... .......... ..........
 R32676  .......... .......... .......... .......... ..........
 T47439  .......... .......... .......... .......... ..........
 R73968  .......... .......... .......... .......... ..........
 H39840  .......... .......... .......... .......... ..........
 H95233  .......... .......... .......... .......... ..........
 H39841  .......... .......... .......... .......... ..........
 N30199  .......... .......... .......... .......... ..........
 T52966  .......... .......... .......... .......... ..........
 N29508  .......... .......... .......... .......... ..........
 N26919  .......... .......... .......... .......... ..........
 N26910  .......... .......... .......... .......... ..........
 H16757  .......... .......... .......... .......... ..........
 N27732  .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

	151				200
Bikunin	GAAAGGCGAC	TTCCGGGGGC	TTTGGCACCT	GGCGGACCCT	CCCGGAGCGT
N40851	GAAAGGCGAC	TTCCGGGGGC	TTTGGCACCT	GGCGGACCCT	CCCGGAGCGT
N39876	GAAAGGCGAC	TTCCGGGGGC	TTTGGCACCT	GGCGGACCCT	CCCGGAGCGT
R87894	GAAAGGCGAC	TTCCGGGGGC	TTTGGCACCT	GGCGGACCCT	CCCGGAGCGT
H16866	GAAAGGCGAC	TTCCGGGGGC	TTTGGCACCT	GGCGGACG.T	CCCGGAGCN.
R34808	GAAAGGCGAC	TTCCGGGGGC	TTTGGCACCT	GGCGGACCCT	CCCGGAGCGT
T66058	....	....	....	...GGACCCT	CCCGGAGCGT
N57450	GAAAGGCGAC	TTCCGGGGGC	TTTGGCACCT	GGCGGACCCT	CCCGGAGCGT
N57374	GAAAGGCGAC	TTCCGGGGGC	TTTGGCACCT	GGCGGACCCT	CCCGGAGCGT
R35464	....	....	....	....	....
H94519	....	....	....	....	....
N39798	....	....	....	....	....
H87300	....	....	....	....	....
R74593	....	....	....	....	....
R31730	....	....	....	....	....
R34701	....	....	....	....	....
H02982	....	....	....	....	....
R32676	....	....	....	....	....
T47439	....	....	....	....	....
R73968	....	....	....	....	....
H39840	....	....	....	....	....
H95233	....	....	....	....	....
H39841	....	....	....	....	....
N30199	....	....	....	....	....
T52966	....	....	....	....	....
N29508	....	....	....	....	....
N26919	....	....	....	....	....
N26910	....	....	....	....	....
H16757	....	....	....	....	....
N27732	....	....	....	....	....

Figure 4C (Con't)

```
         201                                                250
Bikunin  CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
N40851   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTNTG .AGGGGCTTC
N39876   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
R87894   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
H16866   .GGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
R34808   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTNTG GAGGGGCTTC
T66058   CGGCACCTGA ACGCGAGGC. CTCCATTGCG .GTGCGTGTG NAGGGGCTTC
N57450   CGGCACCTGA ACGCGAGGCG CTCCATTGCG CGTGCGTTTG .AGGGGCTTC
N57374   CGGCACCTGA ACGCGAGGC. CTCCATTGC. CGTGCGTTNG .AGGGGCTTC
R35464   .......... .......... .......... .......... ..........
H94519   .......... .......... .......... .......... ..........
N39798   .......... .......... .......... .......... ..........
H87300   .......... .......... .......... .......... ..........
R74593   .......... .......... .......... .......... ..........
R31730   .......... .......... .......... .......... ..........
R34701   .......... .......... .......... .......... ..........
H02982   .......... .......... .......... .......... ..........
R32676   .......... .......... .......... .......... ..........
T47439   .......... .......... .......... .......... ..........
R73968   .......... .......... .......... .......... ..........
H39840   .......... .......... .......... .......... ..........
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   .......... .......... .......... .......... ..........
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
         251                                                  300
Bikunin  CCGCACCT G ATCGCGAGAC CCCAACGGCT GGTGG CGTC GC TG CGCG
N40851   CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG
N39876   CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG
R87894   CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTNG.CGTC GC.TN.CGCG
H16866   CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTNG.CGTC GC.TGGCGCG
R34808   CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGGGCGTC GC.TG.CGCG
T66058   CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG
N57450   CCGCACCT.G ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GCCTG.CGCG
N57374   CCGGAACTTG ATCGCGAGAC CCCAACGGCT GGTGG.CGTC GC.TG.CGCG
R35464   .......... .......... .......... .......... ..........
H94519   .......... .......... .......... .......... ..........
N39798   .......... .......... .......... .......... ..........
H87300   .......... .......... .......... .......... ..........
R74593   .......... .......... .......... .......... ..........
R31730   .......... .......... .......... .......... ..........
R34701   .......... .......... .......... .......... ..........
H02982   .......... .......... .......... .......... ..........
R32676   .......... .......... .......... .......... ..........
T47439   .......... .......... .......... .......... ..........
R73968   .......... .......... .......... .......... ..........
H39840   .......... .......... .......... .......... ..........
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   .......... .......... .......... .......... ..........
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
         301                                                350
Bikunin  TC TCGGCTG AGCT GGCCA TGGCGCANT  GTTGC GGGC T GAGGC GG
N40851   TC.TCGGCTG AGCT.GGNCA TGTCG
N39876   TC.TCGGCTG AGCT.GGCCA TGGCGCACT. G.TGCGGNGC T.GAGGC.G
R87894   TC.TCGGCTG AGCTTGGCCA TGGCGCANT. GTTNC.GGGC T.NAGGC.GG
H16866   TTCTCGGCTG AGCT.GGCCA TGGCGCANT. GTTGC.GNGC T.GAGGC.GG
R34808   TCTTCGGCTG AGCTGGGCCA TGGCGCANTT GTTGC.GGGC T.GAGGC.GG
T66058   TC.TCGGCTG AGCT.GGCCA TGGCGCANT. GTTGC.GNGC T.GAGGC.GG
N57450   TC.TCGGCTG AGCT.GGCCA TGGCGCANT. GGTGC.GGGC TTGAGGC.GG
N57374   TCCTCGGCTG AGCT.GGCCA TGGCGCANT. GGTGCCGNGC T.GAGGCCGG
R35464   .......... .......... .......... .......... ....GGCCGG
H94519   .......... .......... .......... .......... ..........
N39798   .......... .......... .......... .......... ..........
H87300   .......... .......... .......... .......... ..........
R74593   .......... .......... .......... .......... ..........
R31730   .......... .......... .......... .......... ..........
R34701   .......... .......... .......... .......... ..........
H02982   .......... .......... .......... .......... ..........
R32676   .......... .......... .......... .......... ..........
T47439   .......... .......... .......... .......... ..........
R73968   .......... .......... .......... .......... ..........
H39840   .......... .......... .......... .......... ..........
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   .......... .......... .......... .......... ..........
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
         351                                                 400
Bikunin  AC   GG CG     TTTCTCG   CC TGCTGGG A TCGCT GC T CCTCTCT
R87894   ACG.
H16866   AC..CGNCGT TTTTCTTCG. CCTTGCTGGG ATTCGCTTGC TTCCTNTCTG
R34808   ACGCGGNCG. .TTTTTTCGN CCTTGCTGGG ATTCG.TTG. TTNCTCTCTN
T66058   ...CGGNCG. .TTTTCTCG. CC.TGCTGGG A.TCGCT.GC T.CCTCTCT.
N57450   ANN.NGCCG. ..TTTCTCG. CC.TGCTGGG A.TCGCT.GC T.CCTCTCT.
N57374   AG..GGCCGG ..TTTCTCG. CCTTGCTGGG A.TCGCT.GC T.CCTCTCTG
R35464   .....GTCG. ..TTTCTCG. CCTGGCTGGG A.TCGCT.GC T.CCTCTCT.
H94519   .GCNGCGCG. ..TTNNTCG. CN.TGCTGGG A.TCGCT.GC A.CCTCTCT.
N39798   .......... .......... .....CTGGG ANTCGCT.GC T.CCTCTCT.
H87300   .......... .......... .......... .......... ..........
R74593   .......... .......... .......... .......... ..........
R31730   .......... .......... .......... .......... ..........
R34701   .......... .......... .......... .......... ..........
H02982   .......... .......... .......... .......... ..........
R32676   .......... .......... .......... .......... ..........
T47439   .......... .......... .......... .......... ..........
R73968   .......... .......... .......... .......... ..........
H39840   .......... .......... .......... .......... ..........
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   .......... .......... .......... .......... ..........
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
        401                                            450
Bikunin GGGG TCCTG G  CGGCCGA CCGA GAACG CA GCA TCC ACGACTT CT
H16866  GGGGTTCCTG GG.CGGCCGA CCGA.GAACG CA.GCA.TCC AAGAATTTTT
R34808  GGGGTTC.TG GGGNGGCCGA NCGA.GAACG CAAGCA.TTC ACGA.TTT
T66058  GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGANTT.CT
N57450  GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
N57374  GGGG.TCCTG G..CGGCCGA NCGAAGAANG CA.GCAATCC ANGAATTNCT
R35464  GGGG.TCCTG G.CCGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
H94519  GGGG.TCGNG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
N39798  GGGG.TCCTG G..CGGCCGA CCGA.GAACG CA.GCA.TCC ACGACTT.CT
H87300  .......... .......... .......... .......... ..........
R74593  .......... .......... .......... .......... ..........
R31730  .......... .......... .......... .......... ..........
R34701  .......... .......... .......... .......... ..........
H02982  .......... .......... .......... .......... ..........
R32676  .......... .......... .......... .......... ..........
T47439  .......... .......... .......... .......... ..........
R73968  .......... .......... .......... .......... ..........
H39840  .......... .......... .......... .......... ..........
H95233  .......... .......... .......... .......... ..........
H39841  .......... .......... .......... .......... ..........
N30199  .......... .......... .......... .......... ..........
T52966  .......... .......... .......... .......... ..........
N29508  .......... .......... .......... .......... ..........
N26919  .......... .......... .......... .......... ..........
N26910  .......... .......... .......... .......... ..........
H16757  .......... .......... .......... .......... ..........
N27732  .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

	451				500
Bikunin	GCCTGGTGT	CGAAGGT GG	TGGGCAGATG	CCGGG CCTC	CATGCCTA G
H16866	GCC				
T66058	TCCTGGTGTT	CGAAGG			
N57450	GCCTGGTGT.	CGAAGGT.GG	TGGGCAG		
N57374	GCCTGGTGTT	CGAAAGTTGG	TGGGCANATT	CCGGGGCCTT	CATGNCTAAG
R35464	GCCTGGTGT.	CGAAGGT.GG	TGGGCAGATT	CCGGG.CCTC	CATGCCTA.G
H94519	GCCTGGTGT.	CGAAGGT.GG	TGGGCAGATG	CCGGG.CCTC	CATGCCTA.G
N39798	GCCTGGTGT.	CGAAGGT.GG	TGGGCAGATG	CCGGG.CCTC	CATGCCTA.G
H87300	....	....	....	....	....
R74593	....	....	....	....	....
R31730	....	....	....	....	....
R34701	....	....	....	....	....
H02982	....	....	....	....	....
R32676	....	....	....	....	....
T47439	....	....	....	....	....
R73968	....	....	....	....	....
H39840	....	....	....	....	....
H95233	....	....	....	....	....
H39841	....	....	....	....	....
N30199	....	....	....	....	....
T52966	....	....	....	....	....
N29508	....	....	....	....	....
N26919	....	....	....	....	....
N26910	....	....	....	....	....
H16757	....	....	....	....	....
N27732	....	....	....	....	....

Figure 4C (Con't)

```
         501                                                550
Bikunin  G TGGT GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT ATG
 N57374  GTTGGTTGGT ANAATGTNAA TTAANGATTC TTGCAACTGT TTGTGTNATT
 R35464  G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT.ATG
 H94519  G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT.ATG
 N39798  G.TGGT.GGT ACAATGTCAC TGACGGATCC TGCCAGCTGT TTGTGT.ATG
 H87300  .......... .......... .......... .......... ..........
 R74593  .......... .......... .......... .......... ..........
 R31730  .......... .......... .......... .......... ..........
 R34701  .......... .......... .......... .......... ..........
 H02982  .......... .......... .......... .......... ..........
 R32676  .......... .......... .......... .......... ..........
 T47439  .......... .......... .......... .......... ..........
 R73968  .......... .......... .......... .......... ..........
 H39840  .......... .......... .......... .......... ..........
 H95233  .......... .......... .......... .......... ..........
 H39841  .......... .......... .......... .......... ..........
 N30199  .......... .......... .......... .......... ..........
 T52966  .......... .......... .......... .......... ..........
 N29508  .......... .......... .......... .......... ..........
 N26919  .......... .......... .......... .......... ..........
 N26910  .......... .......... .......... .......... ..........
 H16757  .......... .......... .......... .......... ..........
 N27732  .......... .......... .......... .......... ..........

         551                                                600
Bikunin  GGGGCTGTGA   CGGAAACA GCAATAATTA CCTGACCAAG GA GGAGTGC
 N57374  GGGGCTNTTA AACGGAAANA .CAATAATNA CCTGACCAAA GAAGNAAT..
 R35464  GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
 H94519  GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
 N39798  GGGGCTGTGA ..CGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTGC
 H87300  GATTCGGCAC AGGGGAAACA GCAATAATTA CCTGACCAAG GA.GGAGTNC
 R74593  .......... .......... GCAATAATTA CCTGACCAAG GA.GGAGTGC
 R31730  .......... .......... .......... .......... ..........
 R34701  .......... .......... .......... .......... ..........
 H02982  .......... .......... .......... .......... ..........
 R32676  .......... .......... .......... .......... ..........
 T47439  .......... .......... .......... .......... ..........
 R73968  .......... .......... .......... .......... ..........
 H39840  .......... .......... .......... .......... ..........
 H95233  .......... .......... .......... .......... ..........
 H39841  .......... .......... .......... .......... ..........
 N30199  .......... .......... .......... .......... ..........
 T52966  .......... .......... .......... .......... ..........
 N29508  .......... .......... .......... .......... ..........
 N26919  .......... .......... .......... .......... ..........
 N26910  .......... .......... .......... .......... ..........
 H16757  .......... .......... .......... .......... ..........
 N27732  .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
          601                                                 650
Bikunin   CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
R35464    CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
H94519    CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
N39798    CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
H87300    CTCAAGAAAT GTNCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
R74593    CTCAAGAAAT GTGCCACTGT CACAGAGAAT GCCACGGGTG ACCTGGCCAC
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........

          651                                                 700
Bikunin   CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
R35464    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
H94519    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
N39798    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
H87300    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGTGCTCCC AGAAGGCAGG
R74593    CAGCAGGAAT GCAGCGGATT CCTCTGTCCC AAGT.CTCCC AGAAGGCAGG
R31730    .......... .......... .......... .......... ..........
R34701    .......... .......... .......... .......... ..........
H02982    .......... .......... .......... .......... ..........
R32676    .......... .......... .......... .......... ..........
T47439    .......... .......... .......... .......... ..........
R73968    .......... .......... .......... .......... ..........
H39840    .......... .......... .......... .......... ..........
H95233    .......... .......... .......... .......... ..........
H39841    .......... .......... .......... .......... ..........
N30199    .......... .......... .......... .......... ..........
T52966    .......... .......... .......... .......... ..........
N29508    .......... .......... .......... .......... ..........
N26919    .......... .......... .......... .......... ..........
N26910    .......... .......... .......... .......... ..........
H16757    .......... .......... .......... .......... ..........
N27732    .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
         701                                                  750
Bikunin  ATTCT GAAG ACCACTCCAG CGATATGTT  CAACTAT  G AAGAATACTG
R35464   ATTCTTGAAG ACCACTTCAG CGATATGTTT CAANTATTGN AAGAATAATT
H94519   ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
N39798   ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
H87300   ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
R74593   ATTCT.GAAG ACCACTCCAG CGATATGTT. CAACTAT..G AAGAATACTG
R31730   .......... .......... .......... .......... ..........
R34701   .......... .......... .......... .......... ..........
H02982   .......... .......... .......... .......... ..........
R32676   .......... .......... .......... .......... ..........
T47439   .......... .......... .......... .......... ..........
R73968   .......... .......... .......... .......... ..........
H39840   .......... .......... .......... .......... ..........
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   .......... .......... .......... .......... ..........
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........

         751                                                  800
Bikunin   CACCGCCAA CGCAGT CAC TGGGCC TTG CCGTG CAT  CCTT CCCAC
R35464   GCACCGNCAA CGNATT
H94519   GCACCGCCAA CGCATT.CAC TGGGCC..TG C.GTG.CAT. CCTT.CCCAC
N39798   .CACCGCCAA CGCAGT.CAC TGGGGCCTTG C.GTGGAAT. CCTTTCCCAC
H87300   .CACCGCCAA CGCAGTNCAC TGGGCC.TTG C.GTGGCATN CCTT.CCCAC
R74593   .CACCGCCAA CGCAGT.CAC TGGGCC.TTG CCGTG.CAT. CCTT.CCCAC
R31730   .......... .......... .......... .......... ..........
R34701   .......... .......... .......... .......... ..........
H02982   .......... .......... .......... .......... ..........
R32676   .......... .......... .......... .......... ..........
T47439   .......... .......... .......... .......... ..........
R73968   .......... .......... .......... .......... ..........
H39840   .......... .......... .......... .......... ..........
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   .......... .......... .......... .......... ..........
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
        801                                               850
Bikunin GCTGGTACTT T GACGTGGA GA GGAACTC CTG CAATAA CTTCATCTAT
H94519  GCTGGTACTT T.GNCGT
N39798  GCTGGNAATT TNGACGTTGA GAAGGAAC
H87300  GCTNGTACTT T.GACGTGGA GA.GGAACTC CTGGCAATAA CTTCATCTAT
R74593  GCTGGTACTT T.GACGTGGA GA.GGAACTC CTG.CAATAA CTTCATCTAT
R31730  .......... .......... .......... .......... ..........
R34701  .......... .......... .......... .......... ..........
H02982  .......... ........GA GA.GGAACTC CTG.CAATAA CTTCATCTAT
R32676  .......... .......... .......... .........G ATTC..GGAA
T47439  .......... .......... .......... .......... ..........
R73968  .......... .......... .......... .......... ..........
H39840  .......... .......... .......... .......... ..........
H95233  .......... .......... .......... .......... ..........
H39841  .......... .......... .......... .......... ..........
N30199  .......... .......... .......... .......... ..........
T52966  .......... .......... .......... .......... ..........
N29508  .......... .......... .......... .......... ..........
N26919  .......... .......... .......... .......... ..........
N26910  .......... .......... .......... .......... ..........
H16757  .......... .......... .......... .......... ..........
N27732  .......... .......... .......... .......... ..........

        851                                               900
Bikunin GGAGGCT GC CGGGGCAAT  AAGAACAG C TACCGCTC T GAGGAGGCCT
H87300  GGAGGCTTGC CGGGGCAATN AAGAACAGNT TACCGCTCTT TAGGAGGCCT
R74593  GGAGGCT.GC CGGGGCAAT. AAGAACAG.C TACCGCTC.T GAGGAGGCCT
R31730  .......... .......... .......G.C TACCGCTC.T GAGGAGGCCT
R34701  .......... .......... .......... .......... ..........
H02982  GGNGGCT.GC CGGGG.AAT. AAGAACA.NC TACCGCTC.T GAGGAGGCCT
R32676  CGAGGA..GC CGGGGCAAT. AAGAACAG.C TACCGCTC.T GAGGAGGCCT
T47439  .......... .......... .......... .......... ....NGGCCT
R73968  .......... .......... .......... .......... ..........
H39840  .......... .......... .......... .......... ..........
H95233  .......... .......... .......... .......... ..........
H39841  .......... .......... .......... .......... ..........
N30199  .......... .......... .......... .......... ..........
T52966  .......... .......... .......... .......... ..........
N29508  .......... .......... .......... .......... ..........
N26919  .......... .......... .......... .......... ..........
N26910  .......... .......... .......... .......... ..........
H16757  .......... .......... .......... .......... ..........
N27732  .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
         901                                                950
Bikunin   GCA TGCTC CGCTGCTTCC GC                          CA GCAGGA
H87300   .GCA.T.... .
R74593   .GCA.TGCTC CGCTGCTTCC GC........ .......... .CA.GCAGGA
R31730   .GCA.TGCTC CGCTGCTTCC GC........ .......... .CA.GCAGGA
R34701   .......... ......TTCC GC........ .......... .CAAGCAGGA
H02982   .GCG.TGCTC CGCTGCTTCC GCTGTGTGTT CTCTTCCAGG CCA.GCAGGA
R32676   .GCA.TGCTC CGCTGCTTCC GC........ .......... .CA.GCAGGA
T47439   TGCAGTGCTC CGCTGCTTCC GC........ .......... .CA.GCAGGA
R73968   .......... .......... .......... .......... ..........
H39840   .......... .......... .......... .......... ..........
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   .......... .......... .......... .......... ..........
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........

         951                                               1000
Bikunin  GAA TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC  TGG CGGGGC
R74593   GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGGCCGGGGC
R31730   GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
R34701   AAANTCCTCC CCTCCCCCTT GGCTCAAAGG TGGTGGTTCC TGG.CGGGGC
H02982   GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
R32676   GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
T47439   GAA.TCCTCC CCTGCCCCTT GGCTCAAAGG TGGTGGTTC. TGG.CGGGGC
R73968   .......... .......... .......... .......... ....CGGGGC
H39840   .......... .......... .......... .......... ..........
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   .......... .......... .......... .......... ..........
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
         1001                                                 1050
Bikunin  TGTT CGTGA TGGTGTTGAT CC T CTTCC TGGG AGCCT CC ATGGTC
 R74593  TGTTTCGTGA TGGTGTTGAT CCTT..TTCC TGGGGAGCNT CC.ATGGTCT
 R31730  TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGGGAGCCT CC.ATGGTC.
 R34701  TGTT.CGTGA TGGTGTTGAT CCCTCCTTCC CGGG.AGCCT CCCATGGTCC
 H02982  TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTN.
 R32676  TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTC.
 T47439  TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTC.
 R73968  TGTT.CGTGA TGGTGTTGAT CC.T.CTTCC TGGG.AGCCT CC.ATGGTC.
 H39840  .......... .......... .......... .......... ..........
 H95233  .......... .......... .......... .......... ..........
 H39841  .......... .......... .......... .......... ..........
 N30199  .......... .......... .......... .......... ..........
 T52966  .......... .......... .......... .......... ..........
 N29508  .......... .......... .......... .......... ..........
 N26919  .......... .......... .......... .......... ..........
 N26910  .......... .......... .......... .......... ..........
 H16757  .......... .......... .......... .......... ..........
 N27732  .......... .......... .......... .......... ..........

         1051                                                 1100
Bikunin  TACC TGAT  CCGGGTGGCA CGGAGG AAC C AGG AGCG TGCCCTGCGC
 R74593  TAC..TGATT CCGGGTGGCA AGGAGG.AAC C.AGG.AGCG TGCCCTGCGG
 R31730  TACC.TGAT. CCGGGTGGCA CGGAGGGAAC C.AGGGAGCG TGCCCTGCGC
 R34701  TACCCTGAT. CCGGGTGGCA CGGAGG.AAC CCAGG.ANCG TGCCCTGCGC
 H02982  TACC.TGAT. CCGGGTNGCA CGGAGG.AAC C.AGGGAGCG TGCCCTGCGN
 R32676  TACC.TGAT. CCGGGTGGCA CGGAGG.AAC C.AGGGAGCG TGCCCTGCGC
 T47439  TACC.TGAT. CCGGGTNGCA CGGAGG.AAC C.AGG.AGCG TGCCCTGCGC
 R73968  TACC.TGAT. CCGGGTGGCA CGGAGG.AAC C.AGG.AGCG TGCCCTGCGC
 H39840  .......... .......... ...GGG.AAC C.AGG.AGCG TGCCCTGCGC
 H95233  .......... .......... .......... .......... ..........
 H39841  .......... .......... .......... .......... ..........
 N30199  .......... .......... ..GAGGAACC C.ANG.AGCT TCCCCTGCGC
 T52966  .......... .......... .......... .......... ..........
 N29508  .......... .......... .......... .......... ..........
 N26919  .......... .......... .......... .......... ..........
 N26910  .......... .......... .......... .......... ..........
 H16757  .......... .......... .......... .......... ..........
 N27732  .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
         1101                                                  1150
Bikunin  ACCG TCT G GAGCTCCGGA GATGACAAGG  AGCAGCTGG  TGAAGAAC
R74593   ANCG.TCT.G GAGCTTCGGA GATGACAAGG GNT
R31730   ACCG.TCTGG GAGCTCCGGA GATGACAAGG GAGCAGCTGG GTGAAGAAC.
R34701   ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
H02982   ACCG.TCTNG GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
R32676   ACCG.TCTGG GAGCTCCGGA GATGACAAGG GAGCAGCTGG .TGAAGAAC.
T47439   ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
R73968   ACCG.TCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
H39840   ACCGGTCT.G GAGCTCCGGA GATGACAAGG .AGCAGCTGG .TGAAGAAC.
H95233   .......... .......... .......... .......... ..........
H39841   .......... .......... .......... .......... ..........
N30199   ACCG.TCT.G GAGCTCCGGA GATNACAANG .AGCAGCTGN .TGAAGAACC
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......... .......... .......... ..........
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........

         1151                                                  1200
Bikunin  ACATATGT C CTGT GACCG CCCTGT CGC C AAGAGG A CT GGGGAA
R31730   ACATATGTTC CTGTTGACCG NCCTGTTCGC C.AAGAGG.A TTGGGGGAA.
R34701   ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGGAA.
H02982   ACATATGT.C CTGT.GACCG NCCTGTTCGN C.AAGAGG.A CTNGGGGAAA
R32676   ACATATGTTC CTGTTGACCG CCCTGTTCGC C.AAGAGGGA NTGGGGGAA.
T47439   ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGGAA.
R73968   ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.GGGGAA.
H39840   ACATATGT.C CTGT.GACCG CCCTGT.CGC C.AAGAGG.A CT.NGGGAA.
H95233   .......... .......... .......... .......... ..........
H39841   .......... ........C. CCCTGT.CGC CCAAAAGG.A CT.GGGGAA.
N30199   ACATATGT.C CTGT.GACCG CCCTNT.CGC C.AAGAGG.A CT.GGGNAAA
T52966   .......... .......... .......... .......... ..........
N29508   .......... .......CC. CCCTNT.CGC C.AAGAGG.A CT.GGG.AA.
N26919   .......... .......... .......... .......... ..........
N26910   .......... .......... .......... .......... ..........
H16757   .......... .......... .......... .......... ..........
N27732   .......... .......... .......... .......... ..........
```

Figure 4C (Con't)

```
         1201                                               1250
Bikunin   GGGAGGGG  AGACTAT G  TGT GA GCT TTTTTT  AA A TAGA  GG
 R31730  .GGGAGGGGG A
 R34701  .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TA
 H02982  GGGGAGGGG. AGATTAT.G. TGTTGA.GTT TTTTTT..AA ANTAG
 R32676  GGGGAGGGGG AGANTATTGT TGTTGA.GNT TTTTTTTAAA ATTAGGAGGG
 T47439  .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
 R73968  .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
 H39840  .GGGAGGGG. AGACTAT.G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
 H95233  .......... .......... .......... .......... ..........
 H39841  .GGGAGGGGA AAACNAT.G. TGT.GAACCT TTTTTT.AAA A.TAGA..GG
 N30199  .GGGAGGNG. AGACTAT.G. TGT.AA.GCT TTTTTT..AA A.TAGA..GG
 T52966  .......... .......... .......... .......... ..........
 N29508  .GGGAGGGG. AGACTA..G. TGT.GA.GCT TTTTTT..AA A.TAGA..GG
 N26919  .......... .......... .......... .......... ..........
 N26910  .......... .......... .......... .......... ..........
 H16757  .......... .......... .......... .......... ..........
 N27732  .......... .......... .......... .......... ..........

         1251                                               1300
Bikunin  GATTGACTC   GGATTTG A GT GATC A  TTAGGG  CT GAGGTCTGTT
 R32676  GNTTGANTTC GGGNTTTTNA GTTGATCCAT TTAGGGGGNT GAG
 T47439  GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTNTT
 R73968  GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT
 H39840  GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT
 H95233  .......... .......... ........A. TTAGGG..CT GAGGTCTGTT
 H39841  GATTGACTC. .GGATTTG.A GT.GATC.A. TTAGGG..CT GAGGTCTGTT
 N30199  GATTGACTC. .GGATTTGGA GT.GATC.A. TTAGGG..CT GAGGTCTGTT
 T52966  .......... .......... .......... .......... ..........
 N29508  GATTGACTC. .GGATTTG.A GT.GATCNA. TTAGGG..CT GAGGTCTGTT
 N26919  .......... .......... .......... .......... ..........
 N26910  .......... .......... .......... .......... ..........
 H16757  .......... .......... .......... .......... ..........
 N27732  .......... .......... .......... .......... ..........

         1301                                               1350
Bikunin  TCTCTGGGAG GTAGGACGGC TGCTTCC TG G TC TGGCA  GGGATGGG
 T47439  TCTCTNGGAG GTAGGACGA
 R73968  TCTCTGGGAG GTAGGACGGC TGCTTCC.TG GGTCTTGGCA .GGGATGGGG
 H39840  TCTCTGGGAG GTAGGACGGC TGCTTCC.TG G.TC.TGGCA .GGGATGGG.
 H95233  NCTCTGGGAG NTAGGACGGC TGCCTTCCTG G.TC.TGGCA .GGGATGGG.
 H39841  TCNCTGGGAG GTAGGACGGC TGCTCCCCTG G.TC.TGGCA .GGGATGGG.
 N30199  TCTCTGGGAG GTAGGACGGC TGCTTCC.TG G.TC.TGGCA .GGGATGGG.
 T52966  .......... .......... .......... ..TC.TGGCA .GGGATGGG.
 N29508  TCTCTGGGAG GTAGGACGGC TGCTTCA.TG G.TC.TGGCA .GGGATGGG.
 N26919  .......... .......... .......... .......... ..........
 N26910  .......... .......... .......... .......... ..........
 H16757  .......... .......... .........G G.TC.TGGCA .GGGATGGG.
 N27732  .......... .......... .....CCCTG GGTCCTGNCA AGGNATGGGG
```

Figure 4C (Con't)

```
        1351                                              1400
Bikunin TTTG CTTTG G AAATCCTC T AGGAGGCT CCTCCT CGC ATGG CC TG
R73968  TTTG.CTTTG GGAAATCCTC TTNGGAGGCT CCTCCTTCGC ATGGGCCTTG
H39840  TTTG.CTTTG GAGAATCCTC T.ANGAGGCT CCTCCT.CGC ATGG.CC.TG
H95233  TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
H39841  TTTG.CTTTG G.AAANCCNC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N30199  TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCTTCGC ATGG.CC.TG
T52966  TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N29508  TTTG.CTTTG G.AAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N26919  .......... .......... ....GAGGCT CCTCCT.CGC ATGG.CC.TG
N26910  .....CTTTT GNAAATCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
H16757  TTTGCCTTTG G.AAANCCTC T.AGGAGGCT CCTCCT.CGC ATGG.CC.TG
N27732  TTTG.CTTTG G.AAATCCTC TTAGGAGGCT CCTCCT.CGC ATGG.CC.TG

        1401                                              1450
Bikunin CAGT CT GG CAGCAG CCC CGAGTTGTTT  CC TCGCTG ATC GATTTC
R73968  CAGT.CTNGG CAGCANCCCC CGAGTTTTTT TCCTTCGCTG ATCCGATTTC
H39840  CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
H95233  CAGTTCT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
H39841  CAGT.CT.GG CAGCAG.CCC CGAGTTGTTN .CC.TCGCTG ATC.GATNTC
N30199  CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
T52966  CAGT.CT.GG CAGCAG..CC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
N29508  CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
N26919  CAGT.CTTGG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC
N26910  CAGT.CT..G CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ATCGGATTTC
H16757  CAGTNCT.GG CAGCAGACCC CGAGTTGTTT .CC.TCGCTG ATC.GATTTC
N27732  CAGT.CT.GG CAGCAG.CCC CGAGTTGTTT .CC.TCGCTG ANC.GATTTC

        1451                                              1500
Bikunin TTT CCTCCA GGTAG  AGT TTTC TTTG  CTTATGTTGA ATTCCATTGC
R73968  TTTTCCTCCA GGTAAGAATT TTTCTTTT
H39840  TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
H95233  TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
H39841  TTT.CCCCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ANTCCATTGC
N30199  TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
T52966  TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
N29508  TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
N26919  TTT.CCNCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
N26910  TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
H16757  TTTACCCCCA GGTAG..AGT TTTCCTTTGN CTTATGTTGA ATTCCATTGC
N27732  TTT.CCTCCA GGTAG..AGT TTTC.TTTG. CTTATGTTGA ATTCCATTGC
```

Figure 4C (Con't)

```
        1501                                                 1550
Bikunin CTCTTTT CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT GT
 H39840 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTTTGT
 H95233 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 H39841 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N30199 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 T52966 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N29508 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N26919 CTCTTTT.CN CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N26910 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 H16757 CTCTTTTACT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT
 N27732 CTCTTTT.CT CATCACAGAA GTGATGTTGG AATCGTTTCT TTTGTTT.GT

        1551                                                 1600
Bikunin CTGATTTATG G  TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 H39840 CTGATTTATG GGTTTTTTTT AAGTAT
 H95233 CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 H39841 CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 N30199 CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 T52966 CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 N29508 CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 N26919 CTGATTTATG G..TTTTTTT AAGTNTAAAC AAAAGTTTTT TATTAGCATT
 N26910 CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 H16757 CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT
 N27732 CTGATTTATG G..TTTTTTT AAGTATAAAC AAAAGTTTTT TATTAGCATT

        1601                                                 1650
Bikunin CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 H95233 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAA
 H39841 CTGAAAGAAG GAAAGTAAAN TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N30199 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 T52966 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N29508 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N26919 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N26910 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 H16757 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC
 N27732 CTGAAAGAAG GAAAGTAAAA TGTACAAGTT TAATAAAAAG GGGCCTTCCC

        1651                                      1689
Bikunin CTTTAG AAT AAAAAAAAAA AAAAAAAAAA AAAAAAAAA
 H39841 CTTTAA.
 N30199 CTTTAG.AAT AAA
 T52966 CTTTAGGAAT NAAAANAAAA AAGGGTG
 N29508 CTTTAG.AAT AAATTTCAGC ATGTGCTTTC AA
 N26919 CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA A
 N26910 CTTTAG.AAT AAATTTCAGC ATGTGCTTTC AAAAAA
 H16757 CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA AAAAAA
 N27732 CTTTAG.AAT AAAAAAAAAA AAAAAAAAAA AAAAAAAAA
```

FIGURE 4D

```
EST consens MLRAEADGVS RLLGSLLLSG VLAADRERSI HDFCLVSKVV GRCRASMPRW  50
EST consens WYNVTDGSCQ LFVYGGCDGN SNNYLTKEEC LKKCATVTEN ATGDLATSRN 100
EST consens AADSSVPSAP RRQDSEDHSS DMFNYEEYCT ANAVTGPCRA SFPRWYFDVE 150
EST consens RNSCNNFIYG GCRGNKNSYR SEEACMLRCF RQQENPPLPL GSKVVVLAGL 200
EST consens FVMVLILFLG ASMVYLIRVA RRNQERALRT VWSSGDDKEQ LVKNTYVL   248
```

FIGURE 4E

```
cDNA                                                                 ACC   3
translation                                                          T   -47

cDNA         TGATCGCGAG ACCCCAACGG CTGGTGGCGT CGCCTGCGCG TCTCGGCTGA  53
translation  .  S  R  D   P  N  G   W  W  R   R  L  R  V   S  A  E  -30

cDNA         GCTGGCCATG GCGCAGCTGT GCGGGCTGAG GCGGAGCCGG GCGTTTCTCG 103
translation  L  A  M   A  Q  L  C   G  L  R   R  S  R   A  F  L  A-13

cDNA         CCCTGCTGGG ATCGCTGCTC CTCTCTGGGG TCCTGGCGGC CGACCGAGAA 153
translation   L  L  G   S  L  L   L  S  G  V   L  A  A   D  R  E     4

cDNA         CGCAGCATCC ACGACTTCTG CCTGGTGTCG AAGGTGGTGG GCAGATGCCG 203
translation  R  S  I  H   D  F  C   L  V  S   K  V  V  G   R  C  R   21

cDNA         GGCCTCCATG CCTAGGTGGT GGTACAATGT CACTGACGGA TCCTGCCAGC 253
translation   A  S  M   P  R  W  W   Y  N  V   T  D  G   S  C  Q  L  38

cDNA         TGTTTGTGTA TGGGGGCTGT GACGGAAACA GCAATAATTA CCTGACCAAG 303
translation   F  V  Y   G  G  C   D  G  N  S   N  N  Y   L  T  K    54

cDNA         GAGGAGTGCC TCAAGAAATG TGCCACTGTC ACAGAGAATG CCACGGGTGA 353
translation  E  E  C  L   K  K  C   A  T  V   T  E  N  A   T  G  D   71

cDNA         CCTGGCCACC AGCAGGAATG CAGCGGATTC CTCTGTCCCA AGTGCTCCCA 403
translation   L  A  T   S  R  N  A   A  D  S   S  V  P   S  A  P  R  88

cDNA         GAAGGCAGGA TTCTGAAGAC CACTCCAGCG ATATGTTCAA CTATGAAGAA 453
translation   R  Q  D   S  E  D   H  S  S  D   M  F  N   Y  E  E   104

cDNA         TACTGCACCG CCAACGCAGT CACTGGGCCT TGCCGTGCAT CCTTCCCACG 503
translation  Y  C  T  A   N  A  V   T  G  P   C  R  A  S   F  P  R  121

cDNA         CTGGTACTTT GACGTGGAGA GGAACTCCTG CAATAACTTC ATCTATGGAG 553
translation   W  Y  F   D  V  E  R   N  S  C   N  N  F   I  Y  G  G 138

cDNA         GCTGCCGGGG CAATAAGAAC AGCTACCGCT CTGAGGAGGC CTGCATGCTC 603
translation   C  R  G   N  K  N   S  Y  R  S   E  E  A   C  M  L   154

cDNA         CGCTGCTTCC GCCAGCAGGA GAATCCTCCC CTGCCCCTTG GCTCAAAGGT 653
translation  R  C  F  R   Q  Q  E   N  P  P   L  P  L  G   S  K  V  171

cDNA         GGTGGTTCTG GCGGGGCTGT TCGTGATGGT GTTGATCCTC TTCCTGGGAG 703
translation   V  V  L   A  G  L  F   V  M  V   L  I  L   F  L  G  A 188

cDNA         CCTCCATGGT CTACCTGATC CGGGTGGCAC GGAGGAACCA GGAGCGTGCC 753
translation   S  M  V   Y  L  I   R  V  A  R   R  N  Q   E  R  A   204

cDNA         CTGCGCACCG TCTGGAGCTT CGGAGATGA                        782
translation  L  R  T  V   W  S  F   G  D                            213
```

FIGURE 4F

```
cDNA        GCACGAGTTG GGAGGTGTAG CGCGGCTCTG AACGCGCTGA GGGCCGTTGA   50
cDNA        GTGTCGCAGG CGGCGAGGGC GCGAGTGAGG AGCAGACCCA GGCATCGCGC  100
cDNA        GCCGAGAAGG CCGGGCGTCC CCACACTGAA GGTCCGGAAA GGCGACTTCC  150
cDNA        GGGGGCTTTG GCACCTGGCG GACCCTCCCG GAGCGTCGGC ACCTGAACGC  200
cDNA        GAGGCGCTCC ATTGCGCGTG CGCGTTGAGG GGCTTCCCGC ACCTGATCGC  250
cDNA        GAGACCCCAA CGGCTGGTGG CGTCGCCTGC GCGTCTCGGC TGAGCTGGCC  300
cDNA        ATGGCGCAGC TGTGCGGGCT GAGGCGGAGC CGGGCGTTTC TCGCCCTGCT  350
translation M  A  Q  L   C  G  L   R  R  S   R  A  F  L   A  L  L  -11

cDNA        GGGATCGCTG CTCCTCTCTG GGGTCCTGGC GGCCGACCGA GAACGCAGCA  400
translation  G  S  L   L  L  S  G   V  L  A   A  D  R   E  R  S  I    7

cDNA        TCCACGACTT CTGCCTGGTG TCGAAGGTGG TGGGCAGATG CCGGGCCTCC  450
translation  H  D  F   C  L  V   S  K  V  V   G  R  C   R  A  S     23

cDNA        ATGCCTAGGT GGTGGTACAA TGTCACTGAC GGATCCTGCC AGCTGTTTGT  500
translation M  P  R  W   W  Y  N   V  T  D   G  S  C  Q   L  F  V    40

cDNA        GTATGGGGGC TGTGACGGAA ACAGCAATAA TTACCTGACC AAGGAGGAGT  550
translation  Y  G  G   C  D  G  N   S  N  N   Y  L  T   K  E  E  C   57

cDNA        GCCTCAAGAA ATGTGCCACT GTCACAGAGA ATGCCACGGG TGACCTGGCC  600
translation  L  K  K   C  A  T   V  T  E  N   A  T  G   D  L  A     73

cDNA        ACCAGCAGGA ATGCAGCGGA TTCCTCTGTC CCAAGTGCTC CCAGAAGGCA  650
translation T  S  R  N   A  A  D   S  S  V   P  S  A  P   R  R  Q    90

cDNA        GGATTCTGAA GACCACTCCA GCGATATGTT CAACTATGAA GAATACTGCA  700
translation  D  S  E   D  H  S  S   D  M  F   N  Y  E   E  Y  C  T  107

cDNA        CCGCCAACGC AGTCACTGGG CCTTGCCGTG CATCCTTCCC ACGCTGGTAC  750
translation  A  N  A   V  T  G   P  C  R  A   S  F  P   R  W  Y    123

cDNA        TTTGACGTGG AGAGGAACTC CTGCAATAAC TTCATCTATG GAGGCTGCCG  800
translation F  D  V  E   R  N  S   C  N  N   F  I  Y  G   G  C  R   140

cDNA        GGGCAATAAG AACAGCTACC GCTCTGAGGA GGCCTGCATG CTCCGCTGCT  850
translation  G  N  K   N  S  Y  R   S  E  E   A  C  M   L  R  C  F  157

cDNA        TCCGCCAGCA GGAGAATCCT CCCCTGCCCC TTGGCTCAAA GGTGGTGGTT  900
translation  R  Q  Q   E  N  P   P  L  P  L   G  S  K   V  V  V    173

cDNA        CTGGCGGGGC TGTTCGTGAT GGTGTTGATC CTCTTCCTGG GAGCCTCCAT  950
translation L  A  G  L   F  V  M   V  L  I   L  F  L  G   A  S  M   190

cDNA        GGTCTACCTG ATCCGGGTGG CACGGAGGAA CCAGGAGCGT GCCCTGCGCA 1000
translation  V  Y  L  I   R  V  A   R  R  N   Q  E  R   A  L  R  T  207

cDNA        CCGTCTGGAG CTCCGGAGAT GACAAGGAGC AGCTGGTGAA GAACACATAT 1050
translation  V  W  S   S  G  D   D  K  E  Q   L  V  K   N  T  Y    223

cDNA        GTCCTGTGAC CGCCCTGTCG CCAAGAGGAC TGGGGAAGGG AGGGGAGACT 1100
translation V  L  *                                               225
```

FIGURE 4F (Con't)

```
cDNA        ATGTGTGAGC TTTTTTTAAA TAGAGGGATT GACTCGGATT TGAGTGATCA 1150
cDNA        TTAGGGCTGA GGTCTGTTTC TCTGGGAGGT AGGACGGCTG CTTCCTGGTC 1200
cDNA        TGGCAGGGAT GGGTTTGCTT TGGAAATCCT CTAGGAGGCT CCTCCTCGCA 1250
cDNA        TGGCCTGCAG TCTGGCAGCA GCCCCGAGTT GTTTCCTCGC TGATCGATTT 1300
cDNA        CTTTCCTCCA GGTAGAGTTT TCTTTGCTTA TGTTGAATTC CATTGCCTCC 1350
cDNA        TTTTCTCNAT CACAGAAGTG ATGTTGGAAT CGTTTCTTTT GTTTGTCTGA 1400
cDNA        TTTATGGTTT TTTTAAGTAT AAACAAAAGT TTTTTATTAG CATTCTGAAA 1450
cDNA        GAAGGAAAGT AAAATGTACA AGTTTAATAA AAAGGGGCCT TCCCCTTTAG 1500
cDNA        AATAAATTTC CAGCATGTTG CTTTCAAAAA AAAAAAAAAA AAAA
1550
```

FIGURE 4G

```
EST consens                                         MLR AEADGVSRLL GSLLLSGVLA  -1
PCR clone                                       MAQLCGL RRSRAFLALL GSLLLSGVLA  -1
λcDNA clone                                     MAQLCGL RRSRAFLALL GSLLLSGVLA  -1

EST consens ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
PCR clone   ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50
λcDNA clone ADRERSIHDF CLVSKVVGRC RASMPRWWYN VTDGSCQLFV YGGCDGNSNN  50

EST consens YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
PCR clone   YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100
λcDNA clone YLTKEECLKK CATVTENATG DLATSRNAAD SSVPSAPRRQ DSEDHSSDMF 100

EST consens NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
PCR clone   NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150
λcDNA clone NYEEYCTANA VTGPCRASFP RWYFDVERNS CNNFIYGGCR GNKNSYRSEE 150

EST consens ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
PCR clone   ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200
λcDNA clone ACMLRCFRQQ ENPPLPLGSK VVVLAGLFVM VLILFLGASM VYLIRVARRN 200

EST consens QERALRTVWS SGDDKEQLVK NTYVL                            225
PCR clone   QERALRTVWS FGD                                        213
λcDNA clone QERALRTVWS SGDDKEQLVK NTYVL                            225
```

Purification of Placental Bikunin using Superdex 75 Gel-Filtration

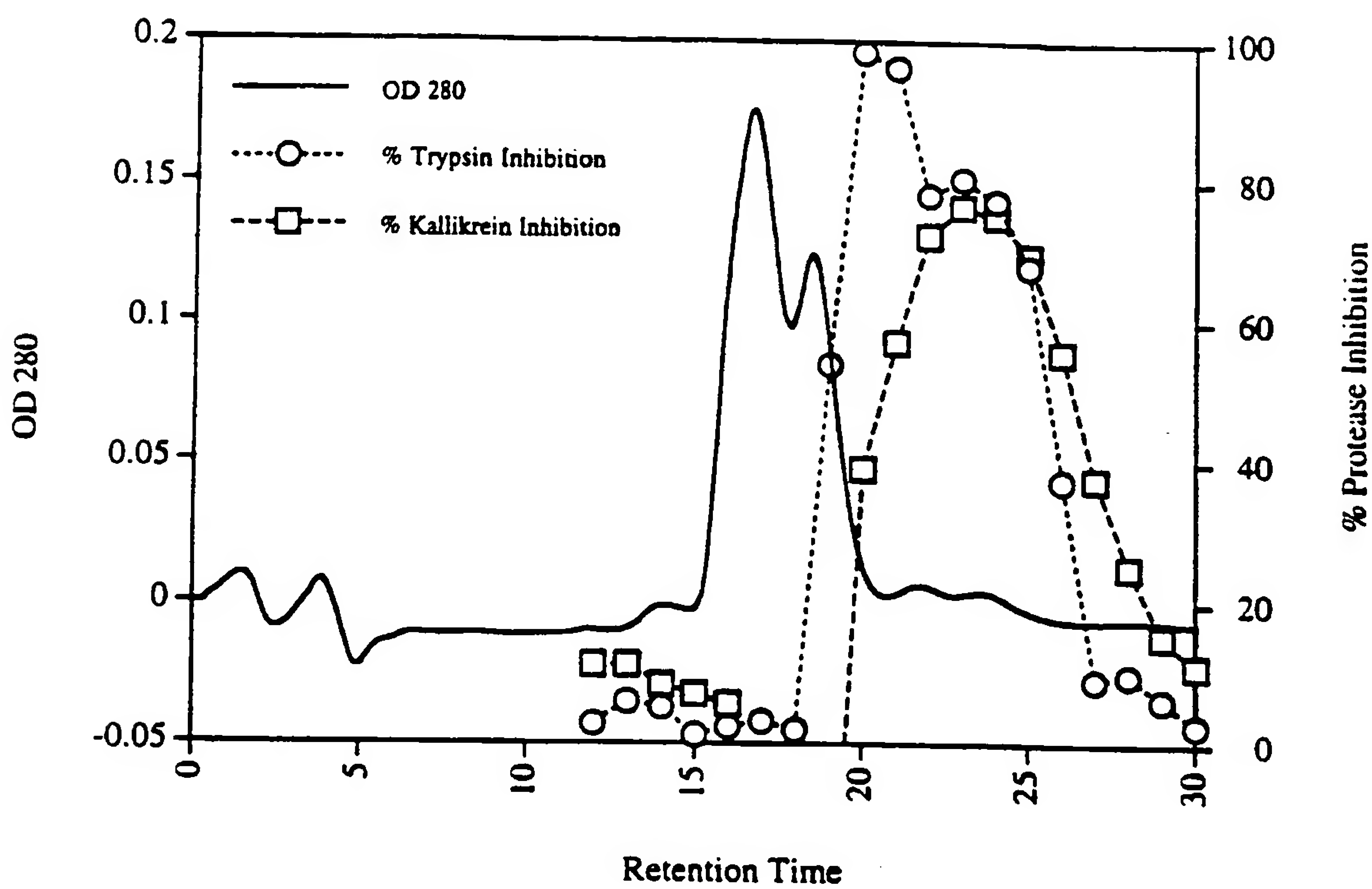


FIGURE 5

FIGURE 6

Purification of Placental Bikunin using C18 Reverse-Phase Chromatography

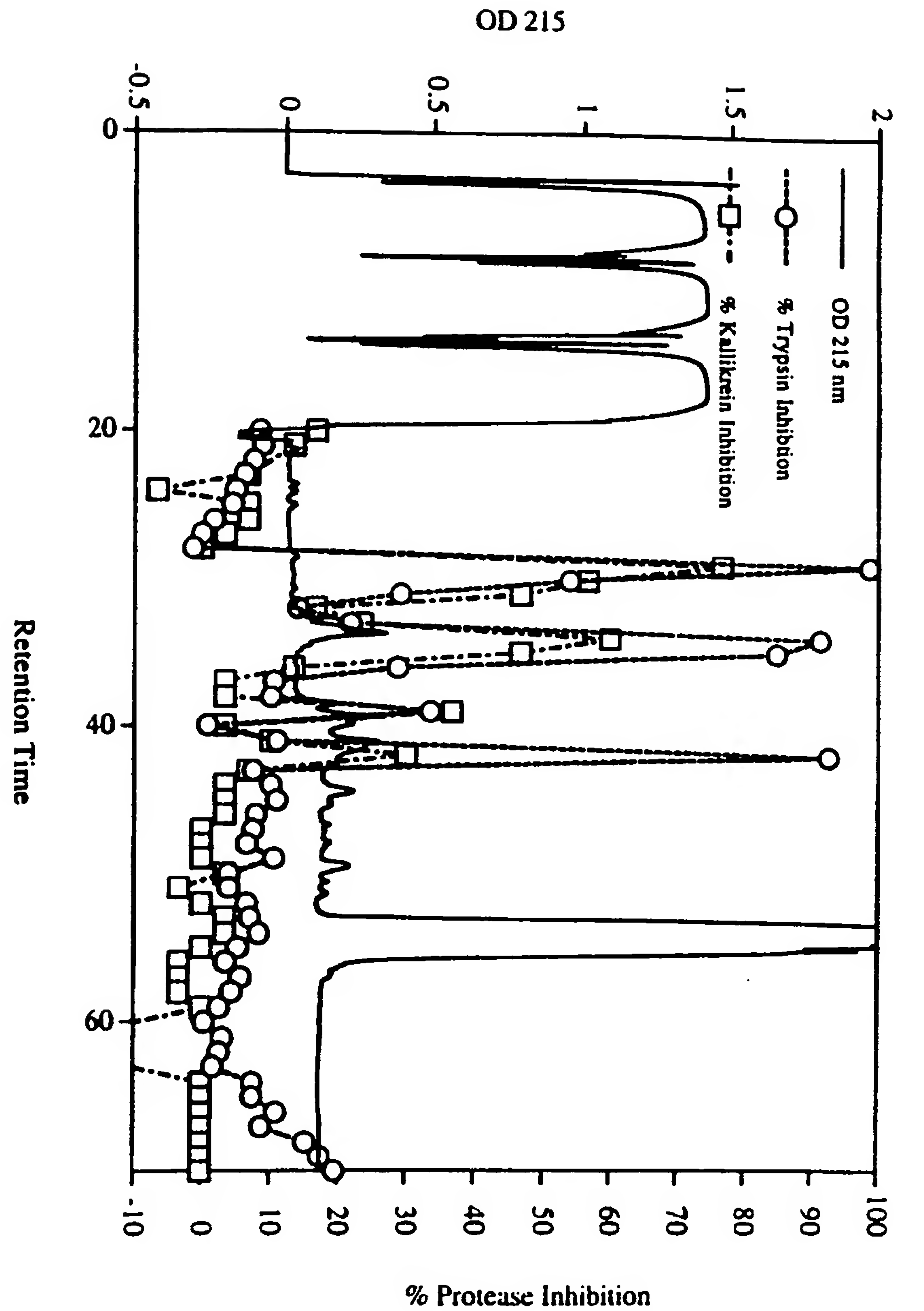


Figure 7

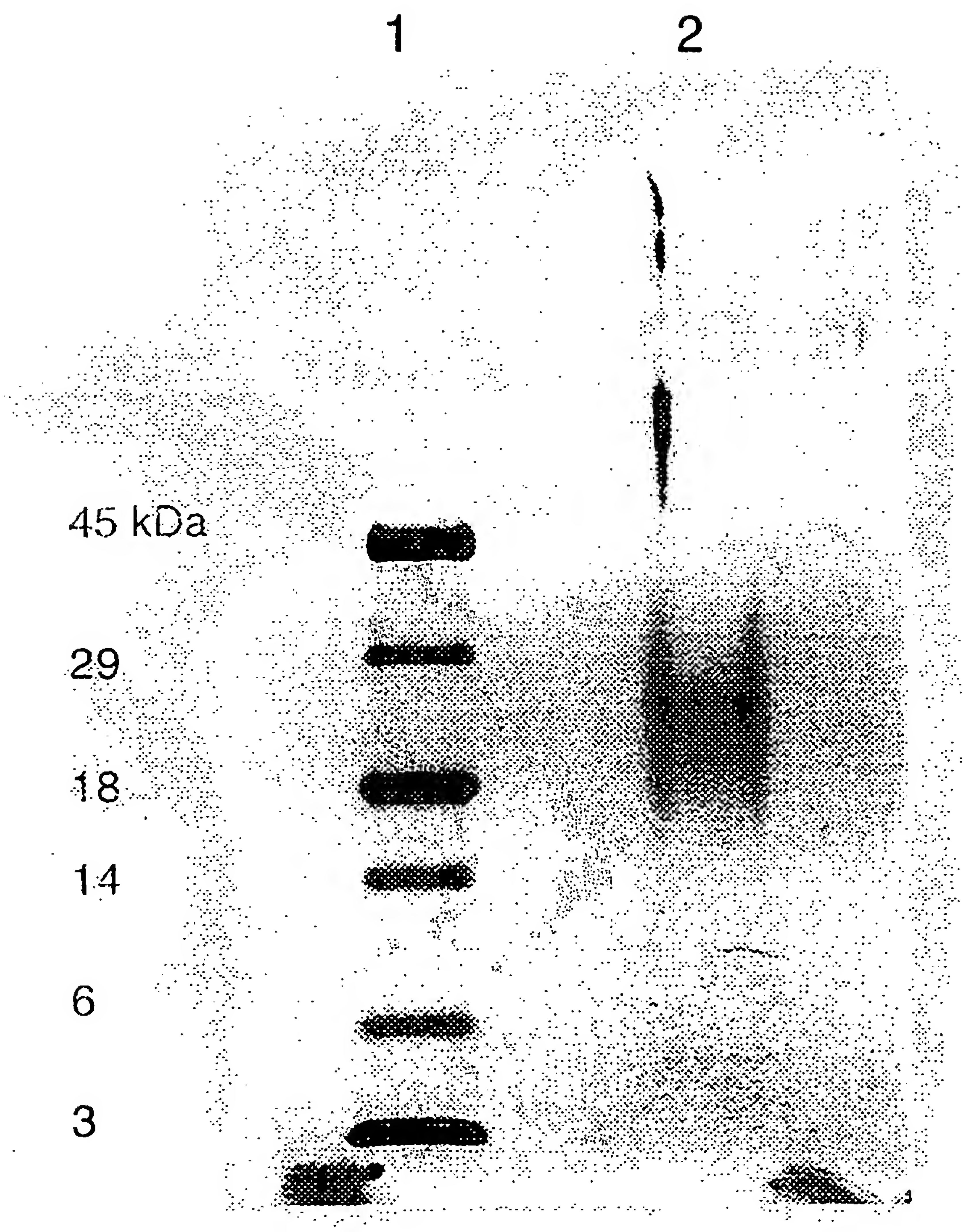


Figure 8A

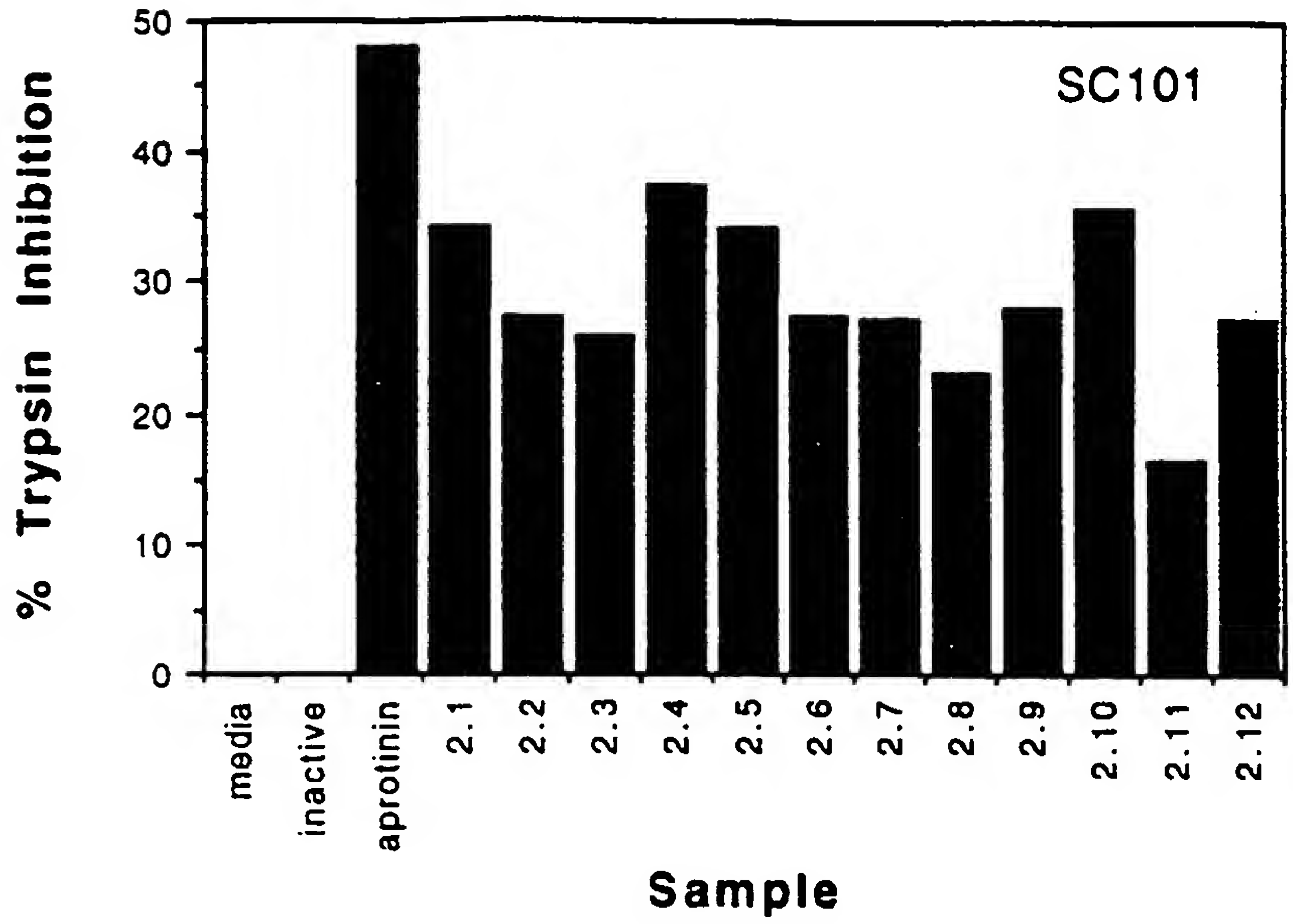


Figure 8B

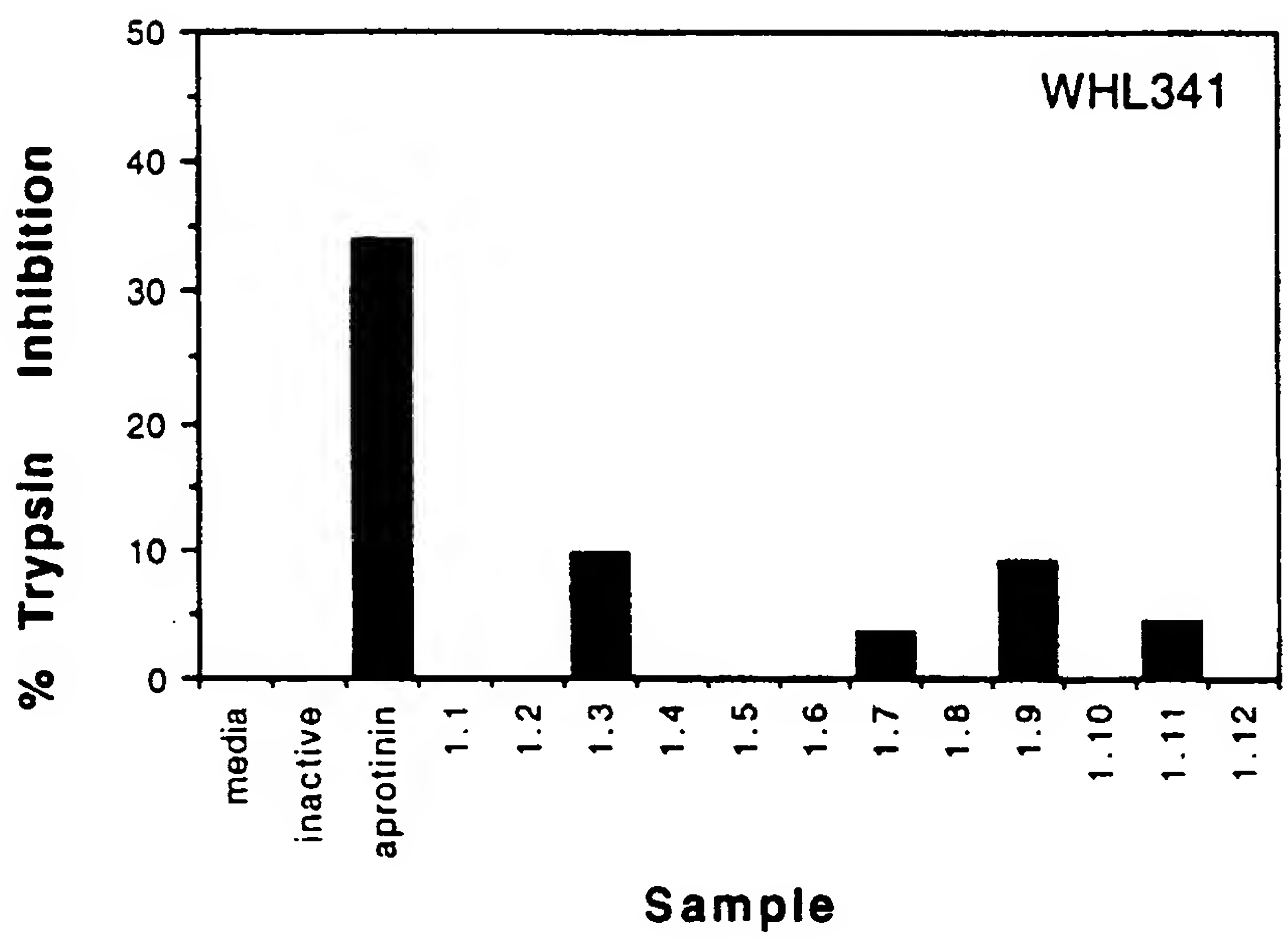


Figure 9A

Figure 9B

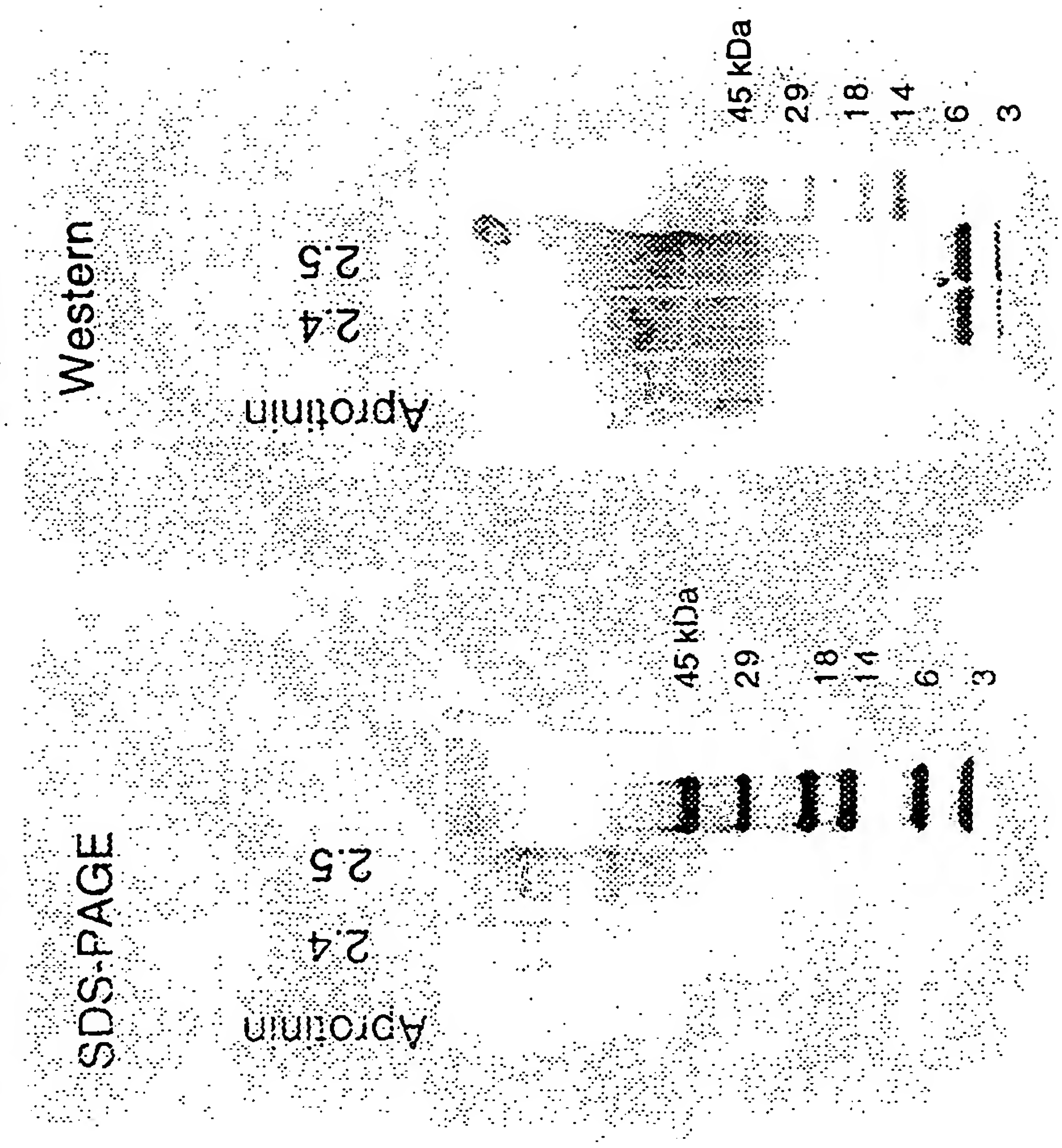


Figure 10

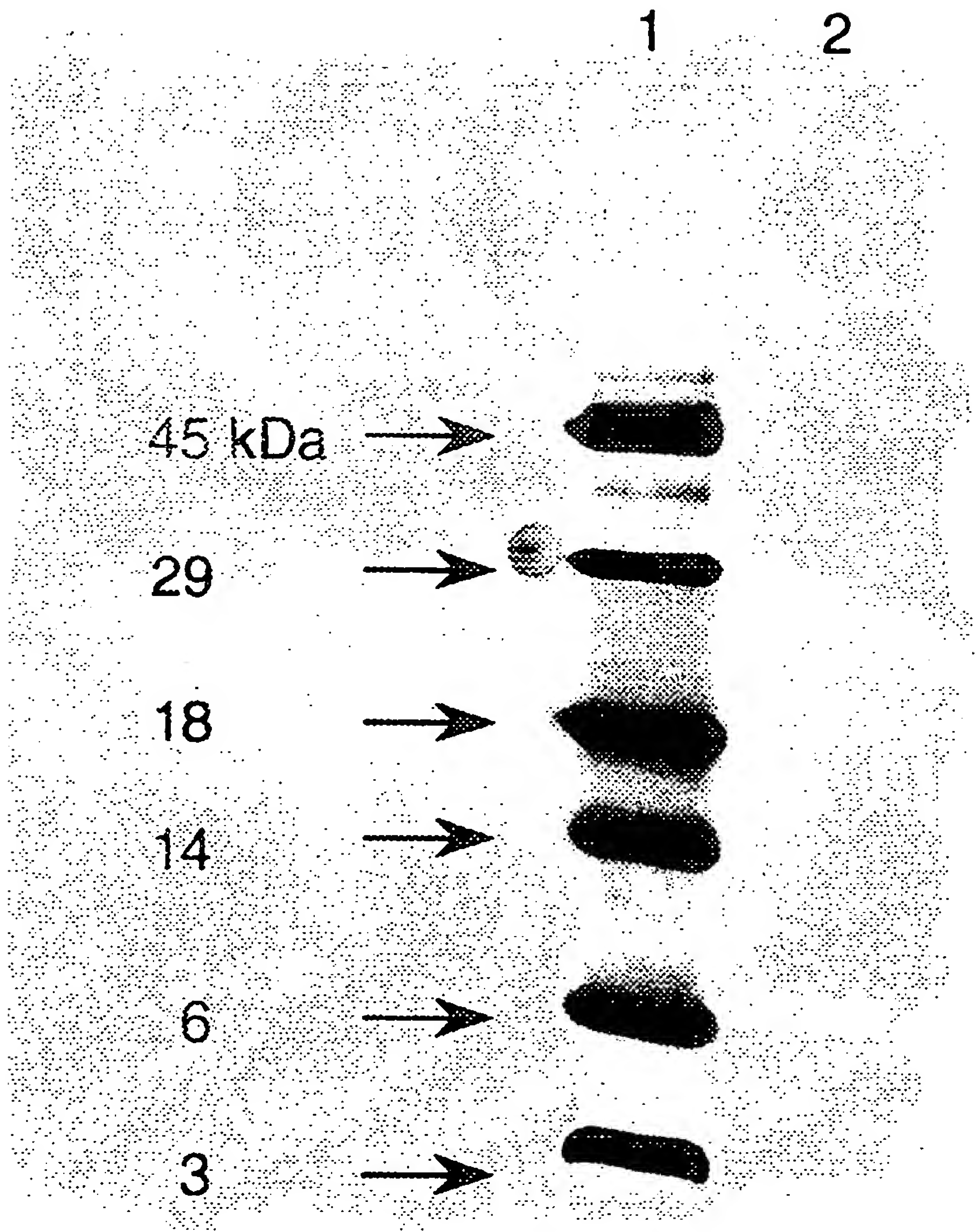


Figure 11B

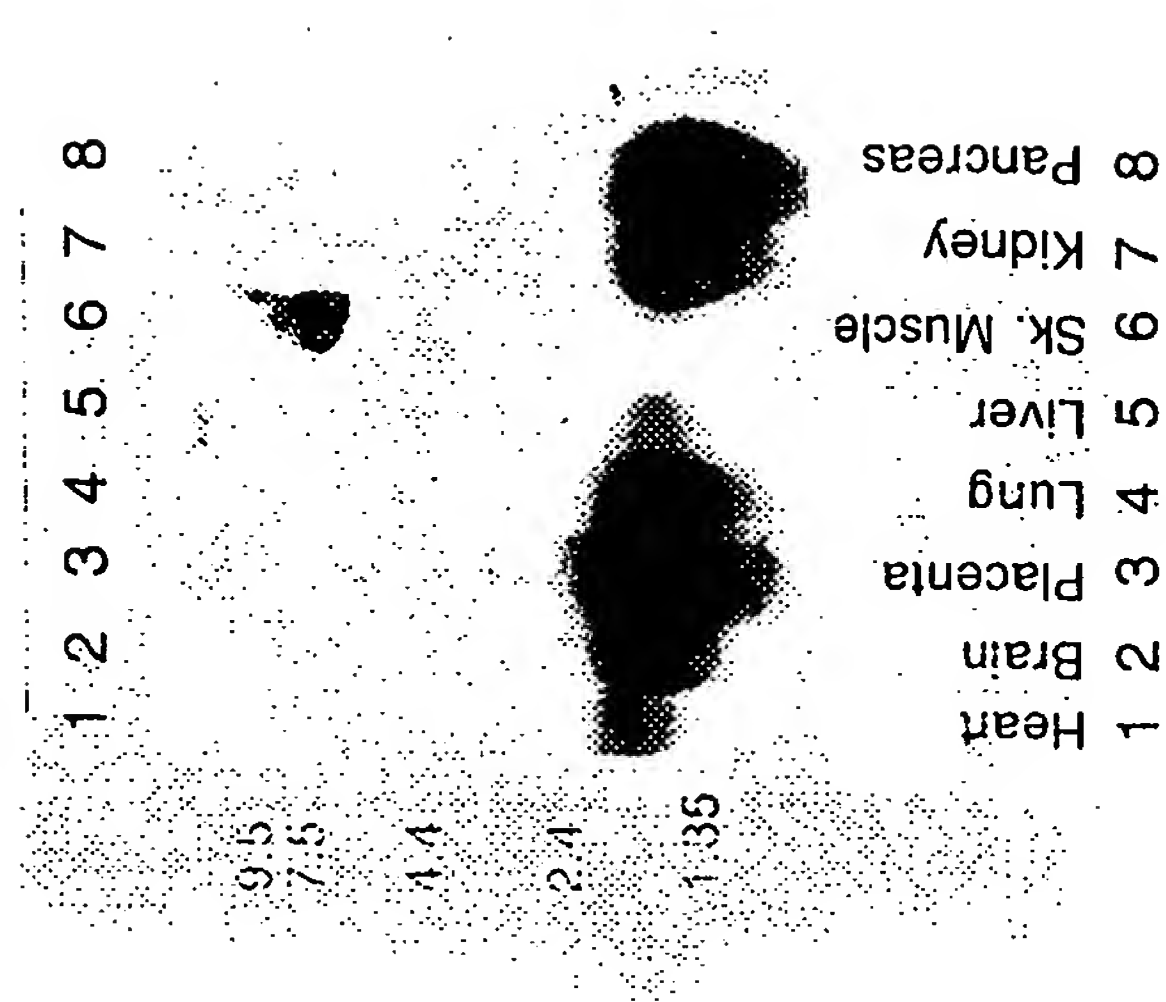
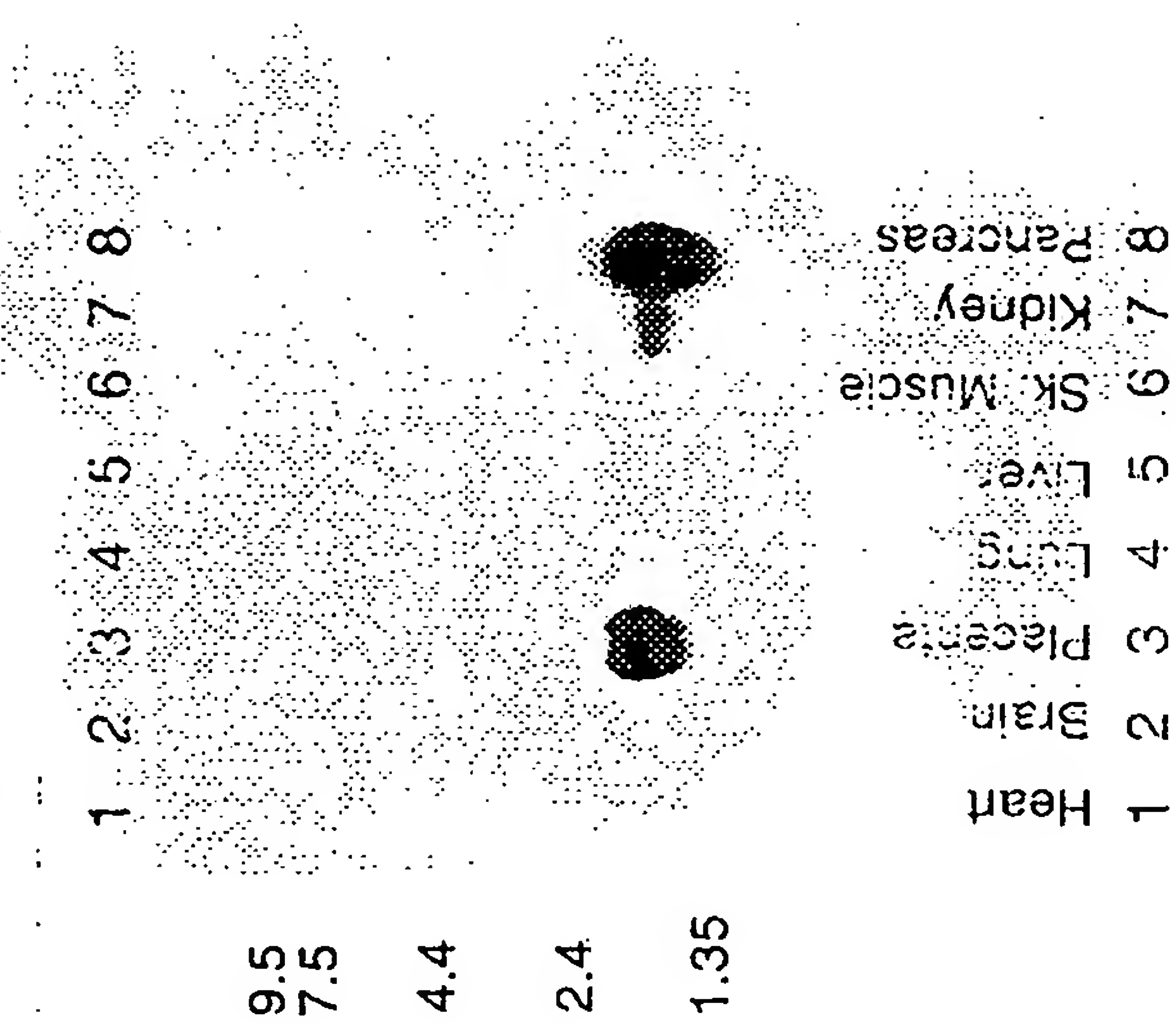


Figure 11A



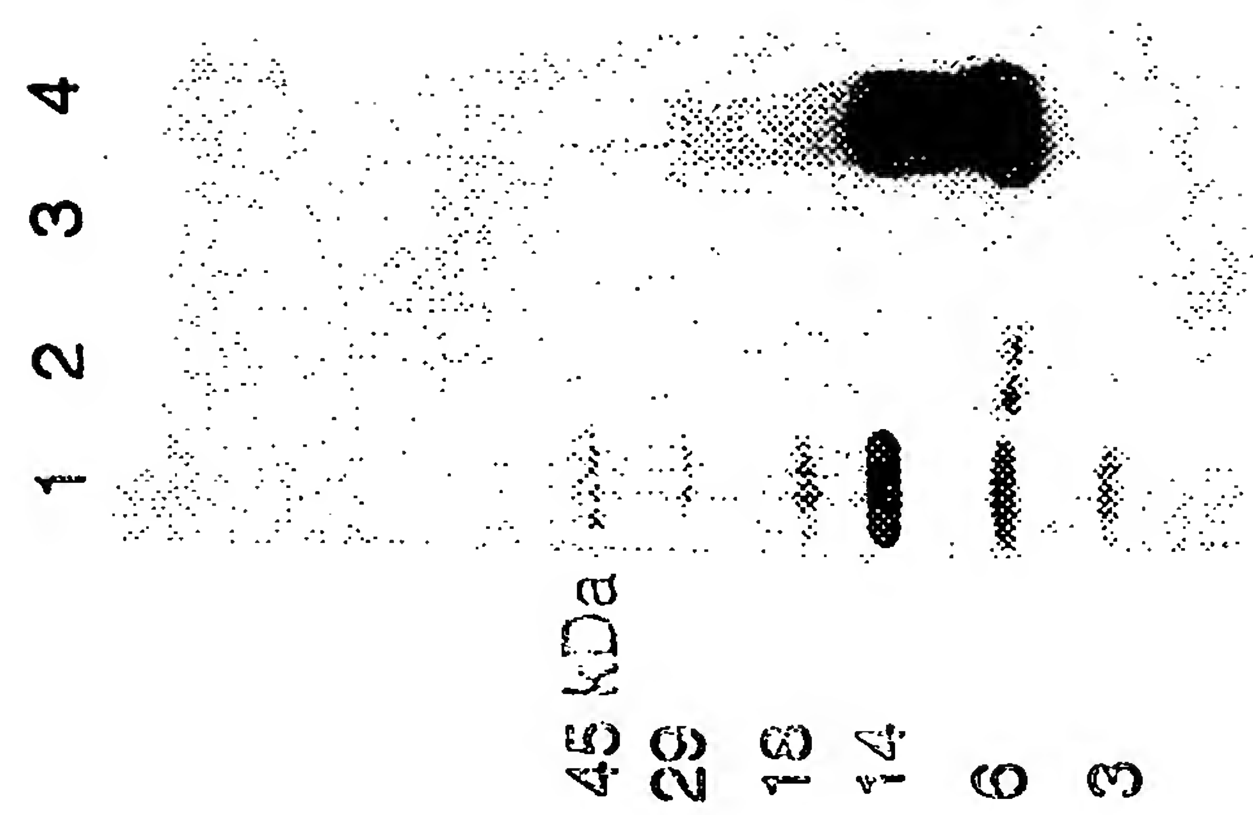


Figure 12B

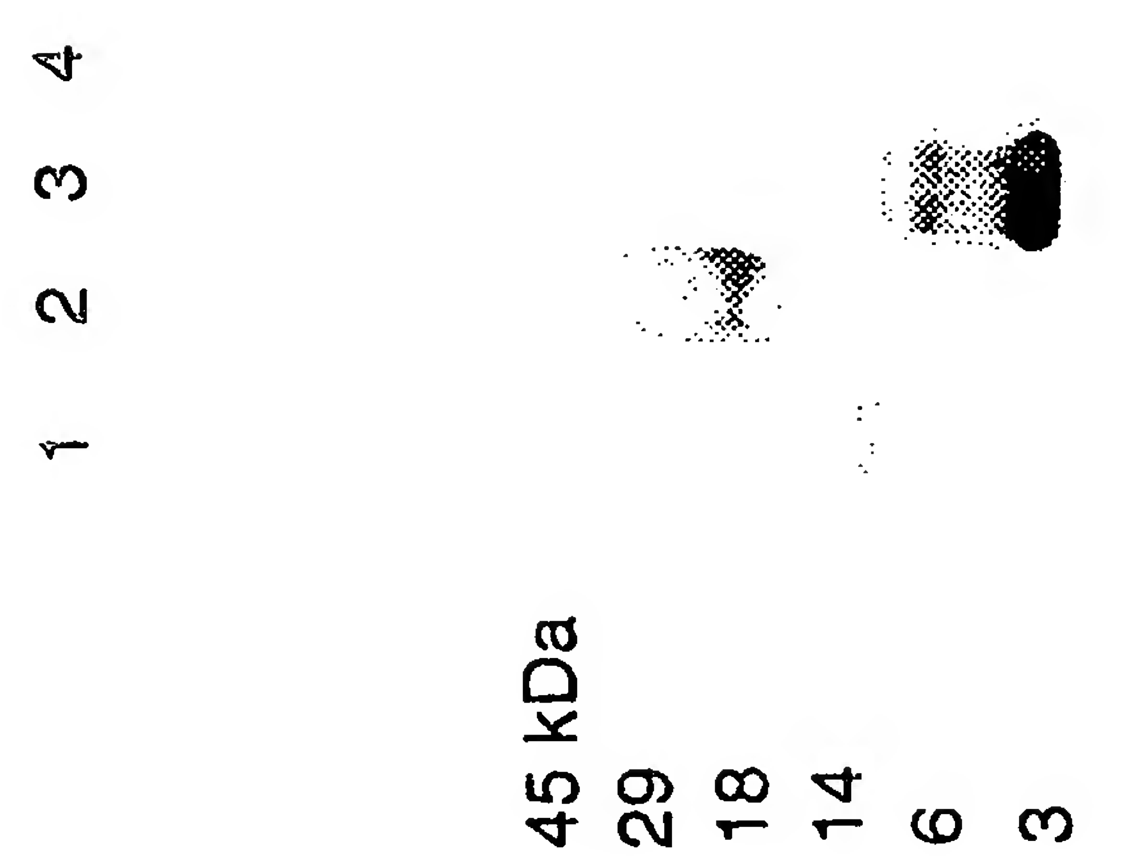


Figure 12A

Figure 13

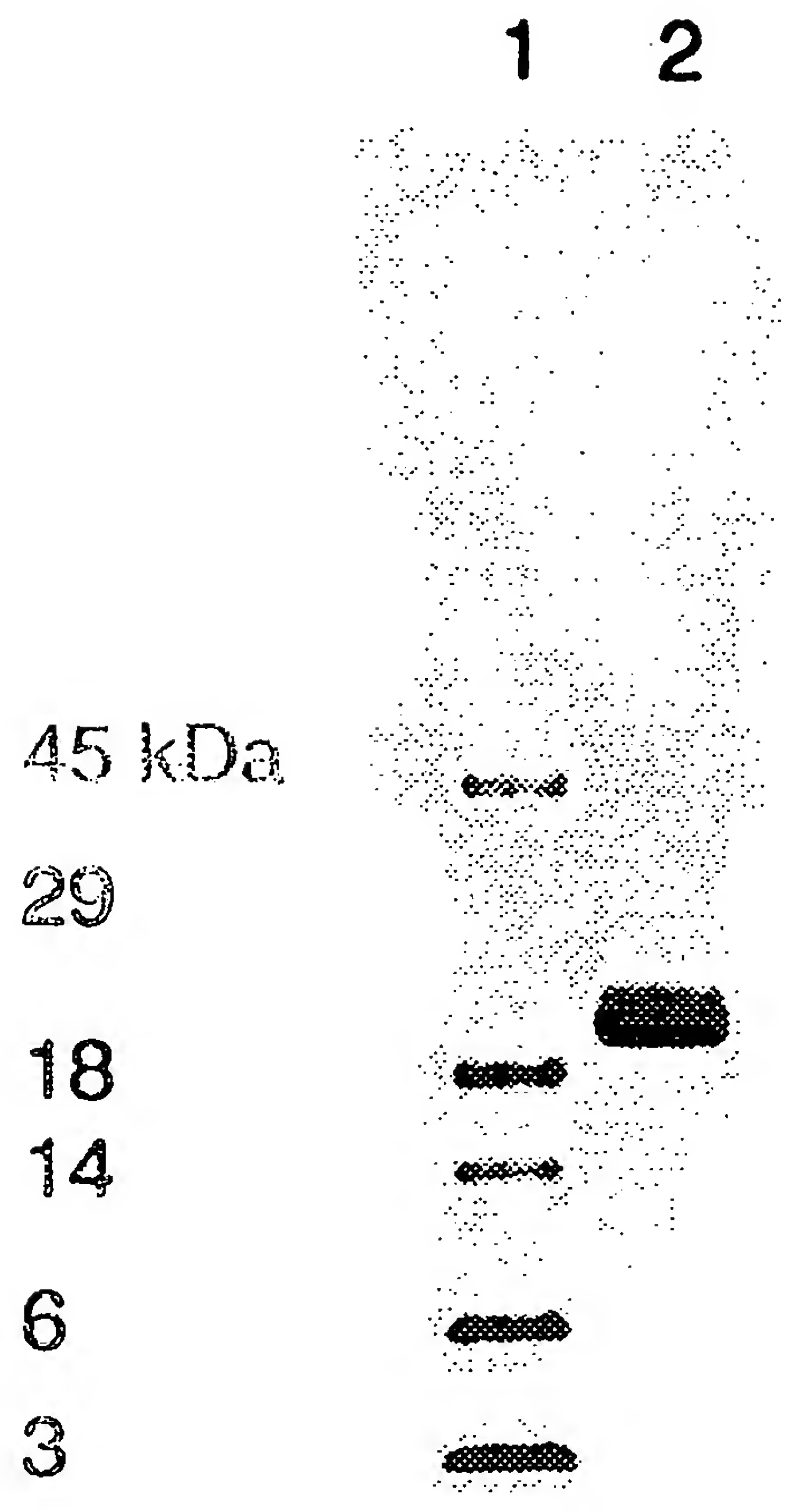
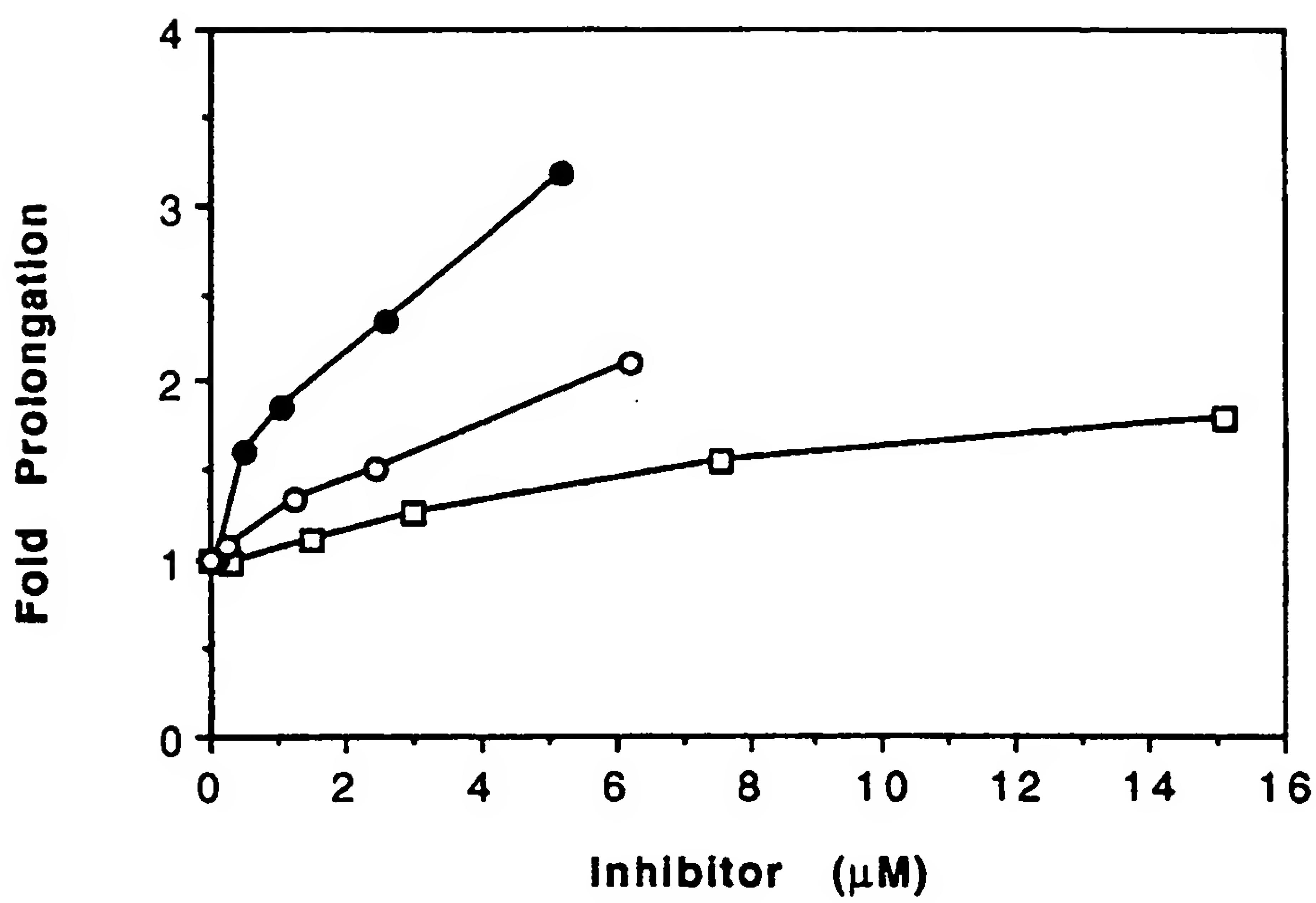


Figure 14



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/03894

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/15 C07K14/81 A61K38/57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL/GENBANK DATABASES Accession no R35464 Sequence reference HS46499, May 4, 1995 L.HILLIER ET AL: "The WashU-Merck EST Project" XP002039653 see the whole document ---	1-6,11
X	EMBL/GENBANK DATABASES Accession no N39798 Sequence reference HS798277, January 26, 1996 L. HILLIER ET AL: "The WasU-Merck EST Project" XP002039654 see the whole document ---	1-6,11

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
4 September 1997	01. 10. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Van der Schaal, C

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL/GENBANK DATABASES Accession no R74593 Sequence reference HS593137,June 9, 1995 L.HILLIER ET AL: "The WashU-Merck EST Project" XP002039655 see the whole document ---	1-6,11
P,X	EP 0 758 682 A (MITSUBISHI CHEM CORP) 19 February 1997 see the whole document ---	1-11
P,A	JOURNAL OF BIOLOGICAL CHEMISTRY 272 (10). 1997. 6370-6376. ISSN: 0021-9258, XP002039700 SHIMOMURA T ET AL: "Hepatocyte growth factor activator inhibitor, a novel Kunitz-type serine protease inhibitor." ---	
A	DATABASE MEDLINE accession no 94289695, 1 July 1994 J. WOJTA ET AL: "Hepatocyte growth factor stimulates expression of plasminogen activator inhibitor type 1 and tissue factor in HepG2 cells" XP002039702 see abstract & BLOOD, vol. 84, no. 1, 1994, pages 151-157, ---	7-10
A	JOURNAL OF BIOLOGICAL CHEMISTRY 271 (7). 1996. 3615-3618. ISSN: 0021-9258, XP002039701 MIYAZAWA K ET AL: "Activation of hepatocyte growth factor in the injured tissues is mediated by hepatocyte growth factor activator." -----	7-10

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/03894

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 7-9
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr   nal Application No
PCT/US 97/03894

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0758682 A	19-02-97	JP 9095498 A	08-04-97

B1

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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60/437,986 6 January 2003 (06.01.2003) US

(71) Applicant *(for all designated States except US)*: **TRANSFERT PLUS** [CA/CA]; 550 Sherbrooke West, Suite 100, H3A 1B9 Montréal, Québec (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BÉLIVEAU, Richard** [CA/CA]; 266 Wilson, H3E 1L8 Montréal, Québec, CA (CA). **DEMEULE, Michel** [CA/CA]; 3557 Archambault, J4M 2W8 Longueuil, Québec, CA (CA).

(74) Agent: **OGILVY RENAULT**; Suite 1600, 1981 McGill College Avenue, H3A 2Y3 Montréal, Québec, CA (CA).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2004/060403 A2

(54) Title: A METHOD FOR TRANSPORTING A COMPOUND ACROSS THE BLOOD-BRAIN BARRIER

(57) Abstract: The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier. The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier.

A METHOD FOR TRANSPORTING A COMPOUND ACROSS THE BLOOD-BRAIN BARRIER

TECHNICAL FIELD

[0001] The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual.

BACKGROUND OF THE INVENTION

[0002] In the development of a new therapy for brain pathologies, the blood-brain barrier (BBB) is considered as a major obstacle for the potential use of drugs for treating disorders of the central nervous system (CNS). The global market for CNS drugs was $33 billion in 1998, which was roughly half that of global market for cardiovascular drugs, even though in the United States, nearly twice as many people suffer from CNS disorders as from cardiovascular diseases. The reason for this lopsidedness is that more than 98% of all potential CNS drugs do not cross the blood-brain barrier. In addition, more than 99% of worldwide CNS drug development is devoted solely to CNS drug discovery, and less than 1% is directed to CNS drug delivery. This ratio could justify why no efficient treatment is currently available for the major neurological diseases such as brain tumors, Alzheimer's and stroke.

[0003] The brain is shielded against potentially toxic substances by the presence of two barrier systems: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB). The BBB is considered to be the major route for the uptake of serum ligands since its surface area is approximately 5000-fold greater than that of BCSFB. The brain endothelium, which constitutes the BBB, represents the major obstacle for the use of potential drugs against many disorders of the CNS. As a general rule, only lipophilic molecules smaller than about 500 Daltons can pass

across the BBB, i.e., from blood to brain. However, the size of many drugs that show promising results in animal studies for treating CNS disorders is considerably bigger. Thus, peptide and protein therapeutics are generally excluded from transport from blood to brain, owing to the negligible permeability of the brain capillary endothelial wall to these drugs. Brain capillary endothelial cells (BCECs) are closely sealed by tight junctions, possess few fenestrae and few endocytic vesicles as compared to capillaries of other organs. BCECs are surrounded by extracellular matrix, astrocytes, pericytes and microglial cells. The close association of endothelial cells with the astrocyte foot processes and the basement membrane of capillaries are important for the development and maintenance of the BBB properties that permit tight control of blood-brain exchange.

[0004] To date, there is no efficient drug delivery approach available for the brain. The methods under investigation for peptide and protein drug delivery to the brain may be divided in three principal strategies. Firstly, invasive procedures include the direct intraventricular administration of drugs by means of surgery, and the temporary disruption of the BBB via intracarotid infusion of hyperosmolar solutions. Secondly, the pharmacologically-based strategy consists in facilitating the passage through the BBB by increasing the lipid solubility of peptides or proteins. Thirdly, physiologic-based strategies exploit the various carrier mechanisms at the BBB, which have been characterized in the recent years. In this approach, drugs are attached to a protein vector that performs like receptors-targeted delivery vehicle on the BBB. This approach is highly specific and presents high efficacy with an extreme flexibility for clinical indications with unlimited targets. In the present invention, the latter approach has been investigated.

[0005] It would be highly desirable to be provided with an improvement in the field of drug delivery.

[0006] It would also be highly desirable to be provided with a non-invasive and flexible method and a carrier for transporting a compound or drug across the BBB of an individual.

SUMMARY OF THE INVENTION

[0007] One aim of the present invention is to provide an improvement in the field of drug delivery.

[0008] Another aim of the present invention is to provide a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual.

[0009] According to one embodiment of the invention, there is provided a method for transporting an agent across the blood-brain barrier of a patient, which comprises the step of administering to the patient a compound comprising the agent attached to aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin.

[0010] According to a further embodiment of the invention, there is provided a use of aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin for transporting a compound attached thereto across the blood-brain barrier of a patient.

[0011] According to another embodiment of the invention, there is provided a use of aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin in the manufacture of a medicament for treating a neurological disease across the blood-brain barrier of a patient.

[0012] According to yet another embodiment of the invention, there is provided a use of aprotinin, a pharmaceutically acceptable salt of aprotinin,

a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin in the manufacture of a medicament for treating a central nervous system disorder across the blood-brain barrier of a patient.

[0013] According to another embodiment of the invention, there is provided compounds of formula R-L-M or pharmaceutically acceptable salts thereof, wherein R is aprotinin or a fragment thereof, L is a linker or a bond and M is an agent or a drug selected from the group consisting of a small molecule drug, a protein, a peptide and an enzyme.

[0014] According to another embodiment of the invention, there is provided a method for treating a neurological disease of a patient comprising administering to the patient a medicament comprising aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin, and a compound adapted to treat the disease, the compound being attached to the aprotinin.

[0015] According to a further embodiment of the invention, there is provided a method for treating a central nervous system disorder of a patient comprising administering to the patient a medicament comprising aprotinin, a pharmaceutically acceptable salt of aprotinin, a fragment of aprotinin or a pharmaceutically acceptable salt of a fragment of aprotinin, and a compound adapted to treat the disease, the compound being attached to the aprotinin.

[0016] In accordance with one embodiment of the present invention, there is provided a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier.

[0017] In a preferred embodiment of the present invention, the transporting does not affect blood-brain barrier integrity.

[0018] In a preferred embodiment of the present invention, the carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.

[0019] In a preferred embodiment of the present invention, the agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

[0020] In a preferred embodiment of the present invention, the anti-cancer agent is Paclitaxel.

[0021] In a preferred embodiment of the present invention, the detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β-galactosidase.

[0022] In a preferred embodiment of the present invention, the agent has a maximum molecular weight of 160,000 Daltons.

[0023] In a preferred embodiment of the present invention, the transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.

[0024] In a preferred embodiment of the present invention, the agent is for treatment of a neurological disease.

[0025] In a preferred embodiment of the present invention, the neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease,

Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunctions.

[0026] In a preferred embodiment of the present invention, the blood-brain barrier related malfunction disease is obesity.

[0027] In a preferred embodiment of the present invention, the transporting results in delivery of the agent to the central nervous system (CNS) of an individual.

[0028] In a preferred embodiment of the present invention, the agent is releasable from the carrier after transport across the blood-brain barrier.

[0029] In a preferred embodiment of the present invention, the agent is released from the carrier after transport across the blood-brain barrier.

[0030] In a preferred embodiment of the present invention, there is provided a pharmaceutical composition for transporting an agent across a blood-brain barrier, the composition comprising a carrier according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0031] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for treating a neurological disease comprising a carrier according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0032] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for delivery of an agent to the CNS of an individual, the composition comprising a carrier according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0033] In accordance with another embodiment of the present invention, there is provided a conjugate for transporting an agent across a blood-brain barrier, the conjugate comprising: (a) a carrier; and (b) an agent attached to the carrier, wherein the conjugate is able to cross the blood-brain barrier and thereby transport the agent across the blood-brain barrier.

[0034] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for transporting an agent across a blood-brain barrier, the composition comprising a conjugate according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0035] In accordance with an embodiment of the present invention, there is provided a pharmaceutical composition for treating a neurological disease, the composition comprising a conjugate according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0036] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for delivery of an agent to the CNS of an individual, the composition comprising a conjugate according to an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0037] In accordance with another embodiment of the present invention, there is provided a use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for transporting the agent across the blood-brain barrier.

[0038] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for transporting an agent across a blood-brain barrier, the composition comprising a medicament

manufactured as defined in an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0039] In accordance with another embodiment of the present invention, there is provided a use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for treating a neurological disease in an individual.

[0040] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for treating a neurological disease comprising a medicament manufactured as defined in an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0041] In accordance with another embodiment of the present invention, there is provided a use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for treating a central nervous system disorder in an individual.

[0042] In accordance with another embodiment of the present invention, there is provided a pharmaceutical composition for treating a central nervous system disorder, the composition comprising a medicament manufactured as defined in an embodiment of the present invention in association with a pharmaceutically acceptable excipient.

[0043] In accordance with another embodiment of the present invention there is provided a conjugate of formula R-L-M or a pharmaceutically acceptable salt thereof, wherein R is a carrier able to cross the blood-brain barrier after attachment to L-M and thereby transport M across the blood-brain barrier, L is a linker or a chemical bond and M is an agent selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level

of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

[0044] In accordance with another embodiment of the present invention, there is provided a use of a conjugate according to an embodiment of the present invention for transporting an agent attached thereto across a blood-brain barrier.

[0045] In accordance with another embodiment of the present invention, there is provided a use of a conjugate according to an embodiment of the present invention for treating a neurological disease in an individual.

[0046] In accordance with another embodiment of the present invention, there is provided a use of a conjugate according to an embodiment of the present invention for treating a central nervous system disorder in an individual.

[0047] In accordance with another embodiment of the present invention, there is provided a method for transporting an agent across a blood-brain barrier, which comprises the step of administering to an individual a pharmaceutical composition according to an embodiment of the present invention.

[0048] In a preferred method of the present invention the pharmaceutical composition is administered to the individual intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*.

[0049] In accordance with another embodiment of the present invention, there is provided a method for treating a neurological disease in an individual comprising administering to the individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to an embodiment of the present invention.

[0050] In accordance with another embodiment of the present invention, there is provided a method for treating a central nervous system disorder in an individual comprising administering to the individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to an embodiment of the present invention.

[0051] For the purpose of the present invention the following terms are defined below.

[0052] The term "carrier" or "vector" is intended to mean a compound or molecule that is able to cross the blood-brain barrier and be attached to or conjugated to another compound or agent and thereby be able to transport the other compound or agent across the blood-brain barrier. For example, the carrier may bind to receptors present on brain endothelial cells and thereby be transported across the blood-brain barrier by transcytosis. Preferably the carrier is a protein or molecule for which very high levels of transendothelial transport are obtained without any effects on the blood-brain barrier integrity. The carrier may be, but is not limited to, a protein, a peptide, or a peptidomimetic and can be naturally occurring or produced by chemical synthesis or recombinant genetic technology (genetic engineering).

[0053] The term "carrier-agent conjugate" is intended to mean a conjugate of a carrier and another compound or agent. The conjugation can be chemical in nature, such as with a linker, or genetic in nature for example by recombinant genetic technology, such as in a fusion protein with for example green fluorescent protein, β-galactosidase or Histag protein.

[0054] The expression "small molecule drug" is intended to mean a drug having a molecular weight of 1000 g/mol or less.

[0055] The terms "treatment", "treating" and the like are intended to mean obtaining a desired pharmacologic and/or physiologic effect, e.g., inhibition of cancer cell growth, death of a cancer cell or amelioration of a neurological disease or condition. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particularly a human, and includes: (a) preventing a disease or condition (e.g., preventing cancer) from occurring in an individual who may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting a disease, (e.g., arresting its development); or (c) relieving a disease (e.g., reducing symptoms associated with a disease). "Treatment" as used herein covers any administration of a pharmaceutical agent or compound to an individual to treat, cure, alleviate, improve, diminish or inhibit a condition in the individual, including, without limitation, administering a carrier-agent conjugate to an individual.

[0056] The term "cancer" is intended to mean any cellular malignancy whose unique trait is the loss of normal controls which results in unregulated growth, lack of differentiation and ability to invade local tissues and metastasize. Cancer can develop in any tissue of any organ. More specifically, cancer is intended to include, without limitation, cancer of the brain.

[0057] The term "administering" and "administration" is intended to mean a mode of delivery including, without limitation, intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*. The preferred one being *per os*. A daily dosage can be divided into one, two or more doses in a suitable form to be administered at one, two or more times throughout a time period.

[0058] The term "therapeutically effective" is intended to mean an amount of a compound sufficient to substantially improve some symptom associated with a disease or a medical condition. For example, in the treatment of cancer or a mental condition or neurological or CNS disease, an agent or compound which decreases, prevents, delays, suppresses, or arrests any symptom of the disease or condition would be therapeutically effective. A therapeutically effective amount of an agent or compound is not required to cure a disease or condition but will provide a treatment for a disease or condition such that the onset of the disease or condition is delayed, hindered, or prevented, or the disease or condition symptoms are ameliorated, or the term of the disease or condition is changed or, for example, is less severe or recovery is accelerated in an individual.

[0059] The carrier and carrier-agent conjugates of the present invention may be used in combination with either conventional methods of treatment and/or therapy or may be used separately from conventional methods of treatment and/or therapy.

[0060] When the carrier-agent conjugates of this invention are administered in combination therapies with other agents, they may be administered sequentially or concurrently to an individual. Alternatively, pharmaceutical compositions according to the present invention may be comprised of a combination of a carrier-agent conjugate of the present invention in association with a pharmaceutically acceptable excipient, as described herein, and another therapeutic or prophylactic agent known in the art.

[0061] It will be understood that a specific "effective amount" for any particular individual will depend upon a variety of factors including the activity of the specific agent employed, the age, body weight, general health, sex, and/or diet of the individual, time of administration, route of

administration, rate of excretion, drug combination and the severity of the particular disease undergoing prevention or therapy.

[0062] Pharmaceutically acceptable acid addition salts may be prepared by methods known and used in the art.

[0063] As used herein, "pharmaceutically acceptable carrier" includes any and all solvents (such as phosphate buffered saline buffers, water, saline), dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

[0064] The term "functional derivative" is intended to mean a "chemical derivative", "fragment", or "variant" biologically active sequence or portion of a carrier or agent or carrier-agent conjugate or a salt thereof of the present invention. A carrier functional derivative is able to be attached to or conjugated to another compound or agent and cross the blood-brain barrier and thereby be able to transport the other compound or agent across the blood-brain barrier.

[0065] The term "chemical derivative" is intended to mean a carrier, an agent, or a carrier-agent conjugate of the present invention, which contains additional chemical moieties not a part of the carrier, agent or carrier-agent conjugate. Covalent modifications are included within the scope of this invention. A chemical derivative may be conveniently prepared by direct chemical synthesis, using methods well known in the art. Such modifications may be, for example, introduced into a protein or peptide carrier, agent or carrier-agent conjugate by reacting targeted amino acid residues with an organic derivatizing agent that is capable of reacting with

selected side chains or terminal residues. A carrier chemical derivative is able to cross the blood-brain barrier and be attached to or conjugated to another compound or agent and thereby be able to transport the other compound or agent across the blood-brain barrier. In a preferred embodiment, very high levels of transendothelial transport across the blood-brain barrier are obtained without any effects on the blood-brain barrier integrity.

[0066] The term "fragment" is intended to mean any piece or portion of a carrier, agent or carrier-agent conjugate. A fragment of a protein or peptide, for example, may be a subset of amino acids which makes up the sequence of the whole protein or peptide. A carrier fragment is able to be attached to or conjugated to another compound or agent and cross the blood-brain barrier and thereby be able to transport the other compound or agent across the blood-brain barrier.

[0067] The term "variant" is intended to mean to a carrier, agent or carrier-agent conjugate which is substantially similar to either the structure of a carrier, agent or carrier-agent conjugate, or any fragment thereof, of the present invention. A carrier variant is able to be attached to or conjugated to another compound or agent and cross the blood-brain barrier and thereby be able to transport the other compound or agent across the blood-brain barrier. Variant proteins, peptides, peptidomimetics and chemical structures of carriers of the present invention are contemplated.

[0068] The term "aprotinin fragment" is intended to mean a portion of aprotinin that can still transport a compound across the BBB. Such a fragment can comprise at least 12 amino acids, preferably at least 25 amino acids and more preferably at least 35 amino acids. Studies to determine the minimal sequence of aprotinin effective to interact with megalin have been performed by Hussain, M., Strickland, D. K., Bakillah, A., in The mammalian low-density lipoprotein receptor family. *Anno. Rev.*

Nutr. 1999, **19**, 141-172. For example, the minimal sequence for interaction of Aprotinin with Megalin receptor was determined to be CRAKRNNFKSA (SEQ ID NO:1). Accordingly, fragments comprising this minimal sequence are meant to be included by this term.

[0069] The term "agent" is intended to mean without distinction a drug or a compound such as a therapeutic agent or compound, a marker, a tracer or an imaging compound.

[0070] The term "therapeutic agent" or "agent" is intended to mean an agent and/or medicine and/or drug used to treat the symptoms of a disease, physical or mental condition, injury or infection and includes, but is not limited to, antibiotics, anti-cancer agents, anti-angiogenic agents and molecules active at the level of the central nervous system Paclitaxel, for example, can be administered intravenously to treat brain cancer.

[0071] The term "patient" or "individual treated" is intended to mean any one who receives a certain medical treatment, and includes being subjected to the administration of a carrier-agent or compound conjugate for detecting, tracing, marking or imaging a condition, such as a tumor. Preferably, the patient or individual treated is a mammal and more preferably a human.

[0072] The term "condition" is intended to mean any situation causing pain, discomfort, sickness, disease or disability (mental or physical) to or in an individual, including neurological disease, injury, infection, or chronic or acute pain. Neurological diseases which can be treated with the present invention include, but are not limited to, brain tumors, brain metastases, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease and stroke.

BRIEF DESCRIPTION OF THE DRAWINGS

[0073] Fig. 1 is a plot showing the results of transcytosis experiments of aprotinin (●), p97 (♦), and ceruloplasmin (■)across bovine brain capillary endothelial cells (BBCECs);

[0074] Fig. 2 is a plot showing the results of transcytosis experiments of aprotinin (●) and transferrin (○) across bovine brain capillary endothelial cells (BBCECs);

[0075] Fig. 3 is a bar graph illustrating that aprotinin has a higher transcytosis capacity than transferrin in a blood-brain barrier model;

[0076] Fig. 4 is an SDS-PAGE analysis illustrating that aprotinin integrity is not affected by its transcytosis across BBCEC monolayers;

[0077] Fig. 5 is a plot of the clearance of [^{14}C]-sucrose expressed as a function of time. The clearance of sucrose was measured in the presence and the absence of 250 nM aprotinin;

[0078] Fig. 6 is a graph showing the results of a sucrose permeability test of bovine brain capillary endothelial cells (BBCECs)

[0079] Fig. 7 is a plot of the clearance of [^{14}C]-sucrose expressed as a function of time illustrating that aprotinin does not affect blood-brain barrier integrity. The clearance of sucrose was measured in the presence and the absence of 5 µM aprotinin;

[0080] Fig. 8 is a bar graph illustrating the accumulation of [^{125}I]-aprotinin in human and rat capillaries;

[0081] Fig. 9 is a plot illustrating a time-course of aprotinin uptake in human and rat capillaries

[0082] Fig. 10 is a bar graph illustrating that aprotinin-biotin conjugate and aprotinin have the same transcytosis capacity;

[0083] Fig. 11 is a bar graph illustrating that aprotinin and aprotinin-biotin conjugate transcytosis is temperature-dependent and conformational-dependent;

[0084] Figs. 12A and 12B are sets of plots illustrating the effect of temperature and heating on (A) aprotinin and (B) aprotinin-biotin conjugate transcytosis in BBCEC cells;

[0085] Fig. 13 is a bar graph illustrating the increase in streptavidin transcytosis in the presence of aprotinin-biotin conjugate;

[0086] Fig. 14 is a bar graph illustrating the inhibition of aprotinin transcytosis by the LRP antagonist, receptor-associated protein (RAP);

[0087] Fig. 15 is a bar graph illustrating aprotinin uptake in an *in situ* brain perfusion experiment;

[0088] Fig. 16 illustrates a synthetic-aprotinin sequence;

[0089] Fig. 17 illustrates a sequence alignment between aprotinin and three human proteins with a similar domain;

[0090] Fig. 18 is a bar graph illustrating *in situ* brain perfusion of transferrin, aprotinin and Angio-pep1;

[0091] Fig. 19 is a plot illustrating transcytosis of Angio-pep1 compared to that of aprotinin; and

[0092] Fig. 20 is a plot illustrating transcytosis of Angio-pep1 across the *in vitro* blood-brain barrier model.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0093] The present invention relates to a new vector or carrier to transport an agent, medicine or other molecule to the brain and/or central nervous system (CNS). This carrier permits the passage of the agent, medicine or other molecule which is attached or coupled (conjugated) to the carrier and which are unable by themselves to cross the blood-brain barrier, to be transported across the blood-brain barrier. The carrier-conjugate can be a carrier-therapeutic agent conjugate. Such conjugates can be in the form of a composition, such as a pharmaceutical composition, for treatment of a condition or disease. This invention is based on the discovery that aprotinin binds to and crosses the brain capillary endothelial wall in a very effective manner. Aprotinin is known in the art to be a basic polypeptide that effectively inhibits a variety of serine proteases, including trypsin, chymotrypsin, kallikrein and pepsin. The transendothelial transport of aprotinin is approximately 10-50 times higher than that of other proteins including transferrin or ceruloplasmin. This high rate of passage is not caused by the disruption of the integrity of the blood-brain barrier since the permeability coefficient for sucrose is not affected by aprotinin.

[0094] This approach is very versatile since it permits conjugation of small as well as large molecules having very diverse therapeutic targets.

[0095] In accordance with the present invention a method for transporting an agent across the blood-brain barrier comprises administering to an individual an agent that comprises an active ingredient or a pharmaceutical agent attached to a carrier, such as aprotinin, or a functional derivative thereof.

[0096] In accordance with the present invention, the compound can be administered intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os* to the patient. The agent is preferably an anti-angiogenic compound. The agent can have

a maximum weight of 160,000 Daltons. Preferably, the agent is a marker or a drug such as a small molecule drug, a protein, a peptide or an enzyme. The drug preferably is adapted to treat a neurological disease or a central nervous system disorder of a patient. The drug can be a cytotoxic drug and the marker can be a detectable label such as a radioactive label, a green fluorescent protein, a histag protein or β-galactosidase. The agent is preferably delivered into the central nervous system of a patient.

[0097] According to still another preferred embodiment of the invention, the uses, methods, compounds, agents, drugs or medicaments of the invention do not alter the integrity of the blood-brain barrier of the patient.

[0098] According to a further preferred embodiment of the invention, aprotinin can be attached to an agent or a compound for transporting the agent or compound across the blood-brain barrier of a patient, the agent or compound being adapted to treat a neurological disease or to treat a central nervous system disorder.

[0099] The carrier or functional derivative thereof of the present invention or mixtures thereof may be linked to or labelled with a detectable label such as a radioimaging agent, such as those emitting radiation, for detection of a disease or condition, for example by the use of a radioimaging agent-antibody-carrier conjugate, wherein the antibody binds to a disease or condition-specific antigen. Other binding molecules besides antibodies and which are known and used in the art are also contemplated by the present invention. Alternatively, the carrier or functional derivative thereof of the present invention or mixtures thereof may be linked to a therapeutic agent, to treat a disease or condition, or may be linked to or labelled with mixtures thereof. Treatment is effected by administering a carrier-agent conjugate of the present invention to an individual under conditions which allow transport of the agent across the blood-brain barrier.

[00100] A therapeutic agent of the present invention can be a drug, a medicine, an agent emitting radiation, a cellular toxin (for example, a chemotherapeutic agent) and/or biologically active fragment thereof, and/or mixtures thereof to allow cell killing or it may be an agent to treat, cure, alleviate, improve, diminish or inhibit a disease or condition in an individual treated. A therapeutic agent can be a synthetic product or a product of fungal, bacterial or other microorganism, such as mycoplasma, viral etc., animal, such as reptile, or plant origin. A therapeutic agent and/or biologically active fragment thereof can be an enzymatically active agent and/or fragment thereof, or can act by inhibiting or blocking an important and/or essential cellular pathway or by competing with an important and/or essential naturally occurring cellular component.

[00101] Radioimaging agents emitting radiation (detectable radio-labels) for use in the present invention are exemplified by indium-111, technitium-99, or low dose iodine-131.

[00102] Detectable labels, or markers, for use in the present invention can be a radiolabel, a fluorescent label, a nuclear magnetic resonance active label, a luminescent label, a chromophore label, a positron emitting isotope for PET scanner, chemiluminescence label, or an enzymatic label. Fluorescent labels include, but are not limited to, green fluorescent protein (GFP), fluorescein, and rhodamine. Chemiluminescence labels include, but are not limited to, luciferase and β-galactosidase. Enzymatic labels include, but are not limited to peroxidase and phosphatase. A histag may also be a detectable label.

[00103] It is contemplated that an agent may be releasable from the carrier after transport across the blood-brain barrier, for example by enzymatic cleavage or breakage of a chemical bond between the carrier and the agent. The release agent would then function in its intended capacity in the absence of the carrier.

[00104] The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

EXPERIMENTAL SECTION

DETERMINATION OF A SUITABLE CARRIER

[00105] A reproducible blood-brain barrier *in vitro* model demonstrating *in vivo* characteristics has been used for screening assay and for mechanistic studies of drug transport to the brain. This efficient *in vitro* model of the blood-brain barrier was developed by the company CELLIAL™ Technologies was of prime importance to the reliable evaluation of the capacity of different carriers to reach the brain. The model consists of a co-culture of bovine brain capillary endothelial cells and rat glial cells. It presents ultrastructural features characteristic of brain endothelium including tight junctions, lack of fenestration, lack of transendothelial channels, low permeability for hydrophilic molecules and a high electrical resistance. Moreover, this model has shown a good correlation coefficient between *in vitro* and *in vivo* analysis of wide range of molecules tested. To date, all the data obtained show that this BBB model closely mimics the *in vivo* situation by reproducing some of the complexities of the cellular environment that exist *in vivo*, while retaining the experimental advantages associated with tissue culture. Thus, many studies have validated this cell co-culture as one of the most reproducible *in vitro* model of the BBB.

[00106] The *in vitro* model of BBB was established by using a co-culture of BBCECs and astrocytes. Prior to cell culture, plate inserts (Millicell-PC 3.0 µM ; 30-mm diameter) were coated on the upper side with rat tail collagen. They were then set in six-well microplates containing the astrocytes and BBCECs were plated on the upper side of the filters in 2 mL of co-culture medium. This BBCEC medium was changed three times a week. Under these conditions, differentiated BBCECs formed a confluent

monolayer 7 days later. Experiments were performed between 5 and 7 days after confluence was reached. The permeability coefficient for sucrose was measured to verify the endothelial permeability.

[00107] Primary cultures of mixed astrocytes were prepared from newborn rat cerebral cortex (Dehouck M.P., Meresse S., Delorme P., Fruchart J.C., Cecchelli, R. An Easier, Reproductible, and Mass-Production Method to Study the Blood-Brain Barrier In Vitro. *J.Neurochem*, 54, 1798-1801, 1990). Briefly, after removing the meninges, the brain tissue was forced gently through a 82 µm nylon sieve. Astrocytes were plated on six-well microplates at a concentration of $1.2x10^5$ cells/mL in 2 mL of optimal culture medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum. The medium was changed twice a week.

[00108] Bovine brain capillary endothelial cells (BBCECs) were obtained from Cellial Technologies. The cells were cultured in the presence of DMEM medium supplemented with 10% (v/v) horse serum and 10% heat-inactivated calf serum, 2 mM of glutamine, 50 µg/mL of gentamycin, and 1 ng/mL of basic fibroblast growth factor, added every other day.

[00109] In order to determine a suitable carrier for the present invention, tests have been performed using the *in vitro* model of the BBB. As illustrated in Fig. 1, transcytosis experiments of different proteins (aprotinin (●), p97 (♦) and ceruloplasmin (■)) across bovine brain capillary endothelial cells (BBCECs) were performed. Figs. 2 and 3 show the results of transcytosis experiments performed with aprotinin (●) and transferrin (○) and using the same method than the experiments of Fig. 1. One insert covered with BBCECs was set into a six-well microplate with 2 mL of Ringer-Hepes and was pre-incubated for 2 h at 37°C. [^{125}I]-aprotinin, [^{125}I]-p97, [^{125}I]-ceruloplasmin or [^{125}I]-transferrin (250 nM final concentration) was added to the upper side of the filter covered with cells. At various times, the insert was transferred to another well to avoid a

possible reendocytosis of [^{125}I]-proteins by the abluminal side of the BBCECs. At the end of experiment, [^{125}I]-proteins were assessed in 500 µL of the lower chamber of well by TCA precipitation. The results indicate that aprotinin has a higher transcytosis capacity than transferrin, p97 or ceruloplasmin in a blood-brain barrier model.

[00110] Aprotinin, p97 and bovine holo-transferrin were iodinated with standard procedures using iodo-beads from Sigma™. Bovine holo-transferrin was diluted in 0.1M phosphate buffer, pH 6.5 (PB). P97 obtained from Synapse Technologies in neutralized citrate at pH 7.0 was dialyzed against this PB. Two iodo-beads were used for each protein. These beads were washed twice with 3 mL of PB on a Whatman™ filter and resuspended in 60 µL of PB. ^{125}I (1 mCi) from Amersham-Pharmacia biotech was added to the bead suspension for 5 minutes at room temperature. The iodination for each protein was initiated by the addition of 100 µg (80-100 µL). After an incubation of 10 minutes at room temperature, the supernatants were applied on a desalting column prepacked with 5 mL of cross-linked dextran from Pierce and ^{125}I-proteins were eluted with 10 mL of PBS. Fractions of 0.5 mL were collected and the radioactivity in 5 µL of each fraction was measured. Fractions corresponding to ^{125}I-proteins were pooled and dialyzed against Ringer-Hepes, pH 7.4. The efficiency of radiolabeling was between 0.6-1 x 10^8 cpm/100 µg of protein.

[00111] From Figs. 1-3, it is clear that aprotinin has a transcytosis capacity which is quite higher than the other tested proteins. The data of Figs. 1-3 have been summarized in Table 1, wherein a comparison of the different proteins has been made.

Table 1

Comparison of ^{125}I-proteins (250 nM) transcytosis across BBCEC monolayers

Proteins compared	Ratios (x-fold)
aprotinin / p97	8.2
aprotinin / ceruloplasmin	44.0
aprotinin / transferrin	11.6

[00112] Table 2 summarizes another experiment, wherein a comparison of additional different proteins has been made.

Table 2

Efficiency of aprotinin to cross the blood-brain barrier

Proteins compared	Transcytosis (pmol/h/cm^2)	Ratios Aprotinin/Protein
Aprotinin	2.7	1
Melanotransferrin (p97)	0.28	10
Transferrin	0.14	19
Lactoferrin	0.05	50
Streptavidin	0.09	30

[00113] In view of Tables 1 and 2, it can be seen that for aprotinin, a superior transendothelial transport was obtained in comparison with the other tested proteins and that the high transcytosis of aprotinin is from about 10 to 50-fold higher than these other proteins.

APROTININ INTEGRITY IS NOT AFFECTED BY ITS TRANSCYTOSIS ACROSS BBCEC MONOLAYERS

[00114] [^{125}I]-protein (0.5-1.5μCi/assay) at a final concentration of 250nM was added to the upper side of filters with or without BBCEC cells placed in 6-well plates. At each time point, filters were put in the next well of the 6-well plates. At the end of the experiment, aliquots were taken in each well and submitted to SDS-PAGE. Gels were then submitted to detection by autoradiography. The results, presented in Fig. 4, indicate that aprotinin integrity is not affected by its transcytosis across BBCEC monolayers.

APROTININ DOES NOT AFFECT THE BLOOD-BRAIN BARRIER INTEGRITY

[00115] A further test was performed to determine the effect of aprotinin at 250 nM on the BBB integrity by measuring [^{14}C] sucrose permeability in the BBB model on BBCEC monolayers grown on filters in the presence of astrocytes. To achieve this test, brain endothelial cell monolayers grown on inserts were transferred to 6-well plates containing 2 mL of Ringer-Hepes per well (basolateral compartment) for two hours at 37 °C. Ringer-Hepes solution was composed of 150 mM NaCl, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 6 mM $NaHC0_3$, 5 mM Hepes, 2.8 mM Hepes, pH 7.4. In each apical chamber, the culture medium was replaced by 1 mL Ringer-Hepes containing the labeled [^{14}C]-sucrose. At different times, inserts were placed into another well. [^{14}C] sucrose passage was

measured at 37°C, on filters without cells (□) or with filters coated with BBCEC cells in the absence (Δ) or presence (o) of 5μM aprotinin (Fig. 6). The results were plotted as the sucrose clearance (μl) as a function of time (min). The sucrose permeability coefficient was then determined. The permeability coefficient (Pe) was calculated as:

1) $\text{Clearance } (\mu l) = \dfrac{[C]A \times VA}{[C]L}$

wherein: [C]A = Abluminal tracer concentration
VA = Volume of abluminal chamber
[C]L = Luminal tracer concentration

2) $1/Pe = (1/PSt - 1/PSf)/\text{filter area } (4.2\ cm^2)$

[00116] At the end of the experiments, amounts of the radiotracers in the basolateral compartment were measured in a liquid scintillation counter. The permeability coefficient (Pe) for sucrose was calculated as previously described (Dehouck, M.P., Jolliet-Riant, P., Brée, F., Fruchart, J.C., Cecchelli, R., Tillement, J.P., *J. Neurochem.* **58**:1790-1797, 1992) using filters coated or non-coated with EC. The results of two experiments were plotted separately in terms of the clearance of [^{14}C]-sucrose (μL) as a function of time (min) (Figs. 5 and 6). In Figs. 5 and 6, PSt represents the permeability x surface area of a filter of the coculture and PSf represents the permeability of a filter coated with collagen and astrocytes plated on the bottom side of the filter B. The permeability coefficient (Pe) was calculated and it was demonstrated that the integrity of the BBB is not affected by aprotinin (see Fig. 6 for Pe calculated from Fig. 5, and Table 3 for Pe calculated from Fig. 7).

Table 3

Permeability coefficients of aprotinin demonstrate that aprotinin does not affect the integrity of the blood-brain barrier

	Pe sucrose (10^{-3} cm/min)
- Aprotinin	0.46 ± 0.09
+ Aprotinin	0.32 ± 0.04

ACCUMULATION OF [^{125}I]-APROTININ IN HUMAN AND RAT CAPILLARIES

[00117] Accumulation was measured at 37°C for 1 hour. Incubation medium contained aprotinin at a final 100 nM concentration in Ringer/Hepes solution. Accumulation was stopped by addition of ice-cold stop-solution and filtration in vacuum through a 0.45μM filter. Nonspecific binding of aprotinin to the capillaries surface was evaluated by the addition of the ice-cold solution before adding the incubation medium. This value was subtracted from accumulation value to obtain the real accumulation value. The results of this experiment are shown in Fig. 8.

TIME-COURSE OF APROTININ UPTAKE IN HUMAN AND RAT CAPILLARIES

[00118] Aprotinin uptake was measured at 37°C for variable time. Incubation medium contained aprotinin at a final 100 nM concentration in Ringer/Hepes solution. At each time point, accumulation was stopped by addition of ice-cold stop-solution and filtration in vacuum through a 0.45μM filter. At each time point, nonspecific binding of aprotinin to the capillaries surface was evaluated by the addition of the ice-cold solution before adding the incubation medium. The results of this experiment are shown in Fig. 9.

APROTININ-BIOTIN CONJUGATE: BIOTINYLATION PROCEDURE

[00119] Water soluble biotin analog Sulfo-NHS-LC-LC-Biotin (Pierce) was used for conjugation. This analog reacts with primary amines in the absence of organic solvent and at neutral pH. A 12-fold molar excess of biotin analog was added to a 10 mg/ml aprotinin solution. Biotin analog and aprotinin mix was incubated for 2 hours at 4°C. To remove unreacted biotin reagent, a dialysis was performed overnight in a slide-a-lyzer dialysis cassette (Pierce) with a 3500 Da cut-off. Determination of biotin incorporation was then performed with the dye HABA (2-(4'-hydroxyazobenzene)-benzoic acid) that binds to avidin yielding an absorption at 500 nm. This binding can be displaced with free biotin or with a biotinylated protein, allowing quantitation of biotin incorporation. The ratio obtained for this conjugation was three biotin for each aprotinin.

APROTININ-BIOTIN CONJUGATE AND APROTININ HAVE THE SAME TRANSCYTOSIS CAPACITY

[00120] Transcytosis of [^{125}I]-aprotinin and [^{125}I]-aprotinin-biotin was evaluated at 37°C. [^{125}I]-protein (0.5-1.5μCi/assay) at a final concentration of 250nM was added to the upper side of the cell-covered filter for transcytosis measurement. At the end of the experiment, [^{125}I]-protein cellular transcytosis was determined directly by TCA precipitation. The results of this experiment are shown in Fig. 10.

APROTININ AND APROTININ-BIOTIN CONJUGATE TRANSCYTOSIS IS TEMPERATURE-DEPENDENT AND CONFORMATIONAL-DEPENDENT

[00121] Accumulation of [^{125}I]-aprotinin and [^{125}I]-aprotinin-biotin was evaluated at 37°C and 4°C, or at 37°C after proteins had been boiled for 10 min at 100°C. [^{125}I]-protein (0.5-1.5μCi/assay) at a final concentration of 250nM was added to the upper side of the cell-covered filter for transcytosis measurement. At the end of the experiment, cell-covered filters were cut and [^{125}I]-protein cellular accumulation was determined directly by TCA precipitation. The results of this experiment are shown in Fig. 11.

EFFECT OF TEMPERATURE AND HEATING ON APROTININ AND APROTININ-BIOTIN CONJUGATE TRANSCYTOSIS IN BBCEC CELLS

[00122] Transcytosis of [^{125}I]-aprotinin (Fig. 12A) and [^{125}I]-aprotinin-biotin (Fig. 12B) was evaluated at 37°C and 4°C, or at 37°C after proteins had been boiled for 10 min at 100°C. [^{125}I]-protein (0.5-1.5μCi/assay) at a final concentration of 250nM was added to the upper side of the cell-covered filter for transcytosis measurement. At each time point filter was moved to the next well of the 6-well plate. At the end of the experiment, [^{125}I]-protein was assessed in the lower compartment of each well by TCA precipitation.

INCREASE IN STREPTAVIDIN TRANSCYTOSIS IN THE PRESENCE OF APROTININ-BIOTIN CONJUGATE

[00123] Transcytosis of [^{125}I]-streptavidin was evaluated alone or in the presence of aprotinin-biotin conjugate. [^{125}I]-protein (0.5-1.5μCi/assay) at a

final concentration of 250nM was added to the upper side of the cell-covered filter for transcytosis measurement. At each time point filter was moved to the next well of the 6-well plate. At the end of the experiment, [^{125}I]-protein was assessed in the lower compartment of each well by TCA precipitation. The results of this experiment are shown in Fig. 13.

INHIBITION OF APROTININ TRANSCYTOSIS BY THE LRP ANTAGONIST, RECEPTOR-ASSOCIATED PROTEIN (RAP)

[00124] Protein transcytosis was evaluated at 37°C. [^{125}I]-aprotinin (0.5-1.5µCi/assay) at a final concentration of 250nM was added to the upper side of the cell-covered filter with or without rap. At the end of the experiment, [^{125}I]-aprotinin was assessed in the lower compartment of each well by TCA precipitation. The results of this experiment are shown in Fig. 14.

APROTININ UPTAKE: *IN SITU* MOUSE BRAIN PERFUSION

Surgical Procedure

[00125] The uptake of [^{125}I]-aprotinin to the luminal side of mouse brain capillaries was measured using the in situ brain perfusion method adapted in our laboratory for the study of drug uptake in the mouse brain (Dagenais et al., 2000, J. Cereb. Blood Flow Metab. 20(2):381-386). Briefly, the right common carotid of ketamine/xylazine (140/8 mg/kg i.p.) anesthetized mice was exposed and ligated at the level of the bifurcation of the common carotid, rostral to the occipital artery. The common carotid was then catheterized rostrally with polyethylene tubing (0.30 mm i.d. × 0.70 mm o.d.) filled with heparin (25 U/ml) and mounted on a 26-gauge needle. The syringe containing the perfusion fluid (10 nM of [^{125}I]-aprotinin in

Krebs/bicarbonate buffer at a pH7.4 gassed with 95% O_2 and 5% CO_2) was placed in an infusion pump (Harvard pump PHD 2000; Harvard Apparatus) and connected to the catheter. Immediately before the perfusion, the heart was stopped by severing the ventricles to eliminate contralateral blood flow contribution. The brain was perfused for 10 min at a flow rate of 2.5ml/min. After 10 min of perfusion, the brain was further perfused for 30 s with Ringer/HEPES solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 6 mM $NaHCO_3$, 5 mM HEPES, 2.8 mM glucose, pH 7.4), to wash the excess of [^{125}I]-aprotinin. Mice were then decapitated to terminate perfusion and the right hemisphere was isolated on ice before being subjected to capillary depletion (Triguero et al., 1990, J Neurochem. 54(6):1882-8). Aliquots of homogenates, supernatants, pellets and perfusates were taken to measure their contents in [^{125}I]-aprotinin by TCA precipitation and to evaluate the apparent volume of distribution.

Determination of BBB transport constants

[00126] Briefly, calculations were carried out as previously described by Smith (1996, Pharm. Biotechnol. 8:285-307). Aprotinin uptake was expressed as the volume of distribution (V_d) from the following equation:

$$Vd=Q^*_{br}/C^*_{pf}$$

where Q^*_{br} is the calculated quantity of [^{125}I]-aprotinin per gram of right brain hemisphere and C^*_{pf} is the labeled tracer concentration measured in the perfusate.

[00127] The results of this experiment, shown in Fig. 15, indicate that there is higher brain uptake for aprotinin than transferrin and that conjugation with biotin does not modify brain uptake of aprotinin.

[00128] In view of the results obtained for the above-mentioned tests, aprotinin is a promising carrier for transporting an agent or compound across the BBB since it has a higher transcytosis across BBCEC monolayers than that of other proteins and it does not alter the integrity of the blood-brain barrier. In addition, aprotinin is not degraded during transcytosis nor does conjugation of aprotinin to biotin affect its transcytosis. Moreover, aprotinin is a versatile and flexible carrier since many molecules such as small drug molecules, proteins, peptides and enzymes may be easily attached to aprotinin proteins for promoting their passage across the BBB. These molecules can conceivably be attached to aprotinin via a linker.

[00129] It has also been determined that the brain distribution volume of aprotinin is higher than that of transferrin. It has further been determined that transcytosis is temperature sensible and conformation dependent, implying that a LDL-R family receptor, probably LRP is involved in aprotinin transcytosis.

[00130] Thus, aprotinin is an effective and efficient carrier to deliver an agent into the brain through the blood-brain barrier.

DESIGN OF A PEPTIDE AS A DRUG VECTOR FOR THE BRAIN

[00131] A sequence comparison was made on the N-terminal sequence of aprotinin (MRPDFCLEPPYTGPCVARIIR) (Fig. 16) (SEQ ID NO:2) using the BLAST™ program on the National Center for Biotechnology Information (NCBI) website. This sequence comparison resulted in four sequences being identified. None of these identified sequences corresponded to a human protein.

[00132] The C-terminal sequence of aprotinin (GLCQTFVYGGCRAKRNNFKSAE) (Fig. 16) (SEQ ID NO:3) was also

compared on the NCBI website. This sequence comparison resulted in 27 sequences being identified with some corresponding to human proteins. The proteins with the highest score were then aligned with the sequence of aprotinin (Fig. 17). From this alignment, the following Angio-pep1 peptide was generated: TFFYGGCRGKRNNFKTEEY (net charge +2) (SEQ ID NO:4).

IN SITU BRAIN PERFUSION OF TRANSFERRIN, APROTININ AND ANGIO-PEP1

[00133] The brain apparent volume of distribution was measured for [^{125}I]-transferrin, [^{125}I]-aprotinin and [^{125}I]-Angio-pep1. Mice brains were perfused for 10 min. Brain capillary depletion was performed to assess the apparent volume of distribution in the brain parenchyma. The results of this experiment are shown in Fig. 18.

TRANSCYTOSIS OF ANGIO-PEP1 COMPARED TO THAT OF APROTININ

[00134] Transcytosis of Angio-pep1 was compared to that of aprotinin. Transport of [^{125}I]-Angio-pep1 and [^{125}I]-aprotinin from the apical-to-basolateral side of endothelial cells monolayers was measured as described above. The final concentration used for angiopep1 and aprotinin for this experiment was 2.5 µM. The results of this experiment are shown in Fig. 19.

TRANSCYTOSIS OF ANGIO-PEP1 ACROSS THE *IN VITRO* BLOOD-BRAIN BARRIER MODEL

[00135] The transport of Angio-pep1 from the apical-to-basolateral side of inserts covered with or without endothelial cell monolayers was measured. The results are expressed as the clearance of Angio-pep1 as a function of time. The slopes correspond to the permeability of the peptide through the filter alone (Psf) and to the total permeability of the endothelial cell monolayers (Pst). The permeability coefficient (Pe) for Angio-pep1 was 1.2 x 10^{-3} cm/min. The results of this experiment are shown in Fig. 20.

[00136] The permeability coefficients for Angio-pep1, aprotinin, leptin and transferrin were determined using the *in vitro* blood-brain barrier model. The permeability coefficient (Pe) was calculated as described above. The comparison of the permeability coefficients is shown in Table 4.

TABLE 4

Permeability coefficients for Angio-pep1, aprotinin, leptin and transferrin

Proteins	Permeability coefficient (Pe) (x 10^{-3} cm/min)	Ratios
Angio-pep1	1.2	1
Aprotinin	0.16	7.5
Leptin	0.055	21
Transferrin	0.0057	210

[00137] The above experiments indicate that brain penetration for Angio-pep1 is higher than that of aprotinin and transferrin. The experiments also indicate that transcytosis of Angio-pep1 measured using the *in vitro* blood-brain barrier model is higher than that of other proteins including aprotinin, leptin and transferrin.

[00138] While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A carrier for transporting an agent attached thereto across a blood-brain barrier, wherein said carrier is able to cross the blood-brain barrier after attachment to said agent and thereby transport said agent across the blood-brain barrier.

2. The carrier according to claim 1, wherein said transporting does not affect blood-brain barrier integrity.

3. The carrier according to claim 1, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.

4. The carrier according to claim 1, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

5. The carrier according to claim 4, wherein said anti-cancer agent is Paclitaxel.

6. The carrier according to claim 4, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β-galactosidase.

7. The carrier according to claim 1, wherein said agent has a maximum molecular weight of 160,000 Daltons.

8. The carrier according to claim 1, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.

9. The carrier according to claim 1, wherein said agent is for treatment of a neurological disease.

10. The carrier according to claim 9, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.

11. The carrier according to claim 10, wherein said blood-brain barrier related malfunction disease is obesity.

12. The carrier according to claim 1, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.

13. The carrier according to claim 1, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.

14. The carrier according to claim 1, wherein said agent is released from said carrier after transport across the blood-brain barrier.

15. A pharmaceutical composition for transporting an agent across a blood-brain barrier, said composition comprising a carrier according to any one of claims 1 to 14 in association with a pharmaceutically acceptable excipient.

16. A pharmaceutical composition for treating a neurological disease, said composition comprising a carrier according to any one of claims 1 to 14 in association with a pharmaceutically acceptable excipient.

17. A pharmaceutical composition for delivery of an agent to the CNS of an individual, said composition comprising a carrier according to any one of claims 1 to 14 in association with a pharmaceutically acceptable excipient.

18. A conjugate for transporting an agent across a blood-brain barrier, said conjugate comprising: (a) a carrier; and (b) an agent attached to said carrier, wherein said conjugate is able to cross said blood-brain barrier and thereby transport said agent across said blood-brain barrier.

19. The conjugate according to claim 18, wherein said transporting does not affect blood-brain barrier integrity.

20. The conjugate according to claim 18, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.

21. The conjugate according to claim 18, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

22. The conjugate according to claim 21, wherein said anti-cancer agent is Paclitaxel.

23. The conjugate according to claim 21, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β-galactosidase.

24. The conjugate according to claim 18, wherein said agent has a maximum molecular weight of 160,000 Daltons.

25. The conjugate according to claim 18, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.

26. The conjugate according to claim 18, for use in treating a neurological disease.

27. The conjugate according to claim 26, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.

28. The conjugate according to claim 27, wherein said blood-brain barrier related malfunction disease is obesity.

29. The conjugate according to claim 18, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.

30. The conjugate according to claim 18, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.

31. The conjugate according to claim 18, wherein said agent is released from said carrier after transport across the blood-brain barrier.

32. A pharmaceutical composition for transporting an agent across a blood-brain barrier, said composition comprising a conjugate according to any one of claims 18 to 31 in association with a pharmaceutically acceptable excipient.

33. A pharmaceutical composition for treating a neurological disease, said composition comprising a conjugate according to any one of claims 18 to 31 in association with a pharmaceutically acceptable excipient.

34. A pharmaceutical composition for delivery of an agent to the CNS of an individual, said composition comprising a conjugate according to any one of claims 18 to 31 in association with a pharmaceutically acceptable excipient.

35. Use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for transporting said agent across said blood-brain barrier.

36. The use according to claim 35, wherein said transporting does not affect blood-brain barrier integrity.

37. The use according to claim 35, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.

38. The use according to claim 35, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

39. The use according to claim 38, wherein said anti-cancer agent is Paclitaxel.

40. The use according to claim 38, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β-galactosidase.

41. The use according to claim 35, wherein said agent has a maximum molecular weight of 160,000 Daltons.

42. The use according to claim 35, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.

43. The use according to claim 35, wherein said carrier is for use in the treatment of a neurological disease.

44. The use according to claim 43, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease,

Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.

45. The use according to claim 44, wherein said blood-brain barrier related malfunction disease is obesity.

46. The use according to claim 35, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.

47. The use according to claim 35, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.

48. The use according to claim 35, wherein said agent is released from said carrier after transport across the blood-brain barrier.

49. A pharmaceutical composition for transporting an agent across a blood-brain barrier, said composition comprising a medicament as defined in any one of claims 35 to 48 in association with a pharmaceutically acceptable excipient.

50. Use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for treating a neurological disease in an individual.

51. The use according to claim 50, wherein said transporting does not affect blood-brain barrier integrity.

52. The use according to claim 50, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.

53. The use according to claim 50, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

54. The use according to claim 53, wherein said anti-cancer agent is Paclitaxel.

55. The use according to claim 53, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β-galactosidase.

56. The use according to claim 50, wherein said agent has a maximum molecular weight of 160,000 Daltons.

57. The use according to claim 50, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.

58. The use according to claim 50, wherein said neurological disease is selected from the group consisting of a brain tumor, a brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.

59. The use according to claim 58, wherein said blood-brain barrier related malfunction disease is obesity.

60. The use according to claim 50, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.

61. The use according to claim 50, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.

62. The use according to claim 50, wherein said agent is released from said carrier after transport across the blood-brain barrier.

63. A pharmaceutical composition for treating a neurological disease. said composition comprising a medicament as defined in any one of claims 50 to 62 in association with a pharmaceutically acceptable carrier.

64. Use of a carrier for transporting an agent attached thereto across a blood-brain barrier in the manufacture of a medicament for treating a central nervous system disorder in an individual.

65. The use according to claim 64, wherein said transporting does not affect blood-brain barrier integrity.

66. The use according to claim 64, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.

67. The use according to claim 64, wherein said agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

68. The use according to claim 67, wherein said anti-cancer agent is Paclitaxel.

69. The use according to claim 67, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β-galactosidase.

70. The use according to claim 64, wherein said agent has a maximum molecular weight of 160,000 Daltons.

71. The use according to claim 64, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.

72. The use according to claim 64, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.

73. The use according to claim 64, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.

74. The use according to claim 64, wherein said agent is released from said carrier after transport across the blood-brain barrier.

75. A pharmaceutical composition for treating a central nervous system disorder, said composition comprising a medicament as defined in any one of claims 64 to 74 in association with a pharmaceutically acceptable excipient.

76. Conjugates of formula R-L-M or a pharmaceutically acceptable salt thereof, for transporting M across a blood-brain barrier wherein R is a carrier able to cross said blood-brain barrier after attachment to

L-M and thereby transport M across said blood-brain barrier, L is a linker or a chemical bond and M is an agent is selected from the group consisting of a drug, a medicine, a protein, a peptide, an enzyme, an antibiotic, an anti-cancer agent, a molecule active at the level of the central nervous system, a radioimaging agent, an antibody, a cellular toxin, a detectable label and an anti-angiogenic compound.

77. The conjugate according to claim 76, wherein said transporting does not affect blood-brain barrier integrity.

78. The conjugate according to claim 76, wherein said carrier is selected from the group consisting of aprotinin, a functional derivative of aprotinin, Angio-pep1 and a functional derivative of Angio-pep1.

79. The conjugate according to claim 76, wherein said detectable label is selected from the group consisting of a radioactive label, a green fluorescent protein, a histag protein and β-galactosidase.

80. The conjugate according to claim 76, wherein said agent has a maximum molecular weight of 160,000 Daltons.

81. The conjugate according to claim 76, wherein said transporting is effected by receptor-mediated transcytosis or adsorptive-mediated transcytosis.

82. The conjugate according to claim 76, wherein M is an agent useful for treating a neurological disease.

83. The conjugate according to claim 82, wherein said neurological disease is selected from the group consisting of a brain tumor, a

brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and blood-brain barrier related malfunction disease.

84. The use according to claim 83, wherein said blood-brain barrier related malfunction disease is obesity.

85. The conjugate according to claim 76, wherein said transporting results in delivery of said agent to the central nervous system (CNS) of an individual.

86. The conjugate according to claim 76, wherein said agent is releasable from said carrier after transport across the blood-brain barrier.

87. The conjugate according to claim 76, wherein said agent is released from said carrier after transport across the blood-brain barrier.

88. A pharmaceutical composition for transporting an agent across a blood-brain barrier, said composition comprising a conjugate according to any one of claims 76 to 87 in association with a pharmaceutically acceptable excipient.

89. A pharmaceutical composition for treating a neurological disease, said composition comprising a conjugate according to any one of claims 76 to 87 in association with a pharmaceutically acceptable excipient.

90. A pharmaceutical composition for delivery of an agent to the CNS of an individual, said composition comprising a conjugate according

to any one of claims 76 to 87 in association with a pharmaceutically acceptable excipient.

91. Use of a conjugate according to any one of claims 18 to 31 and 76 to 87 for transporting an agent attached thereto across a blood-brain barrier.

92. Use of a conjugate according to any one of claims 18 to 31 and 76 to 87 for treating a neurological disease in an individual.

93. Use of a conjugate according to any one of claims 18 to 31 and 76 to 87 for treating a central nervous system disorder in an individual.

94. A method for transporting an agent across a blood-brain barrier, which comprises the step of administering to an individual a pharmaceutical composition according to any one of claims 15, 32, 49 and 88.

95. The method of claim 94, wherein said pharmaceutical composition is administered to said individual intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*.

96. The method of claim 94, wherein said pharmaceutical composition is administered to said individual *per os*.

97. A method for treating a neurological disease in an individual comprising administering to said individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to any one of claims 16, 33, 63 and 89.

98. The method of claim 97, wherein said pharmaceutical composition is administered to said individual intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*.

99. The method of claim 97, wherein said pharmaceutical composition is administered to said individual *per os*.

100. A method for treating a central nervous system disorder in an individual comprising administering to said individual in need thereof a therapeutically effective amount of a pharmaceutical composition according to any one of claims 17, 34, 75 and 90.

101. The method of claim 100, wherein said pharmaceutical composition is administered to said individual intra-arterially, intra-nasally, intra-peritoneally, intravenously, intramuscularly, sub-cutaneously, transdermally or *per os*.

102. The method of claim 100, wherein said pharmaceutical composition is administered to said individual *per os*.

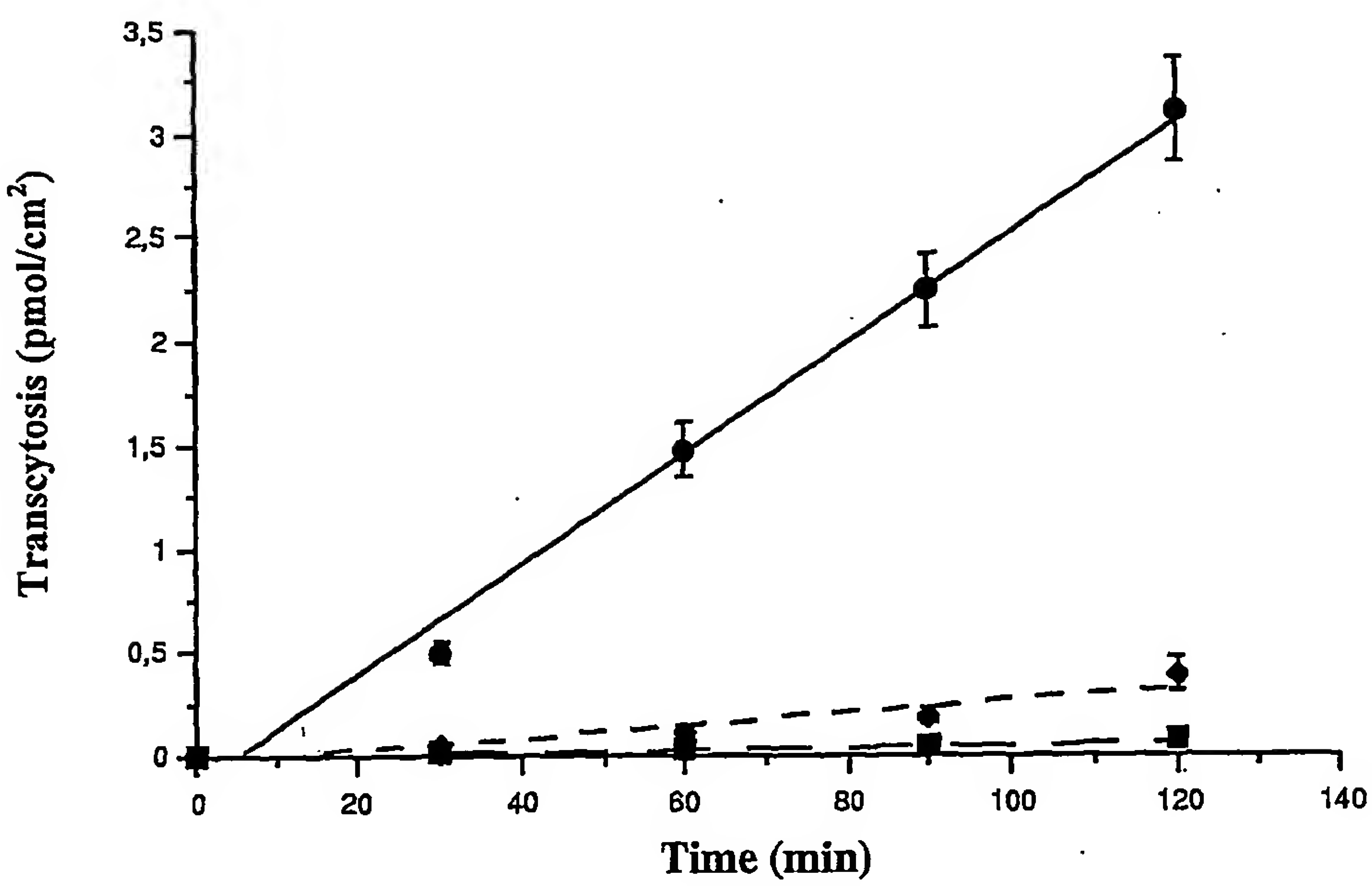

3,5
3
2,5
2
1,5
1
0,5
0
Transcytosis (pmol/cm^2)
0
20
40
60
80
100
120
140
Time (min)

FIG. 1

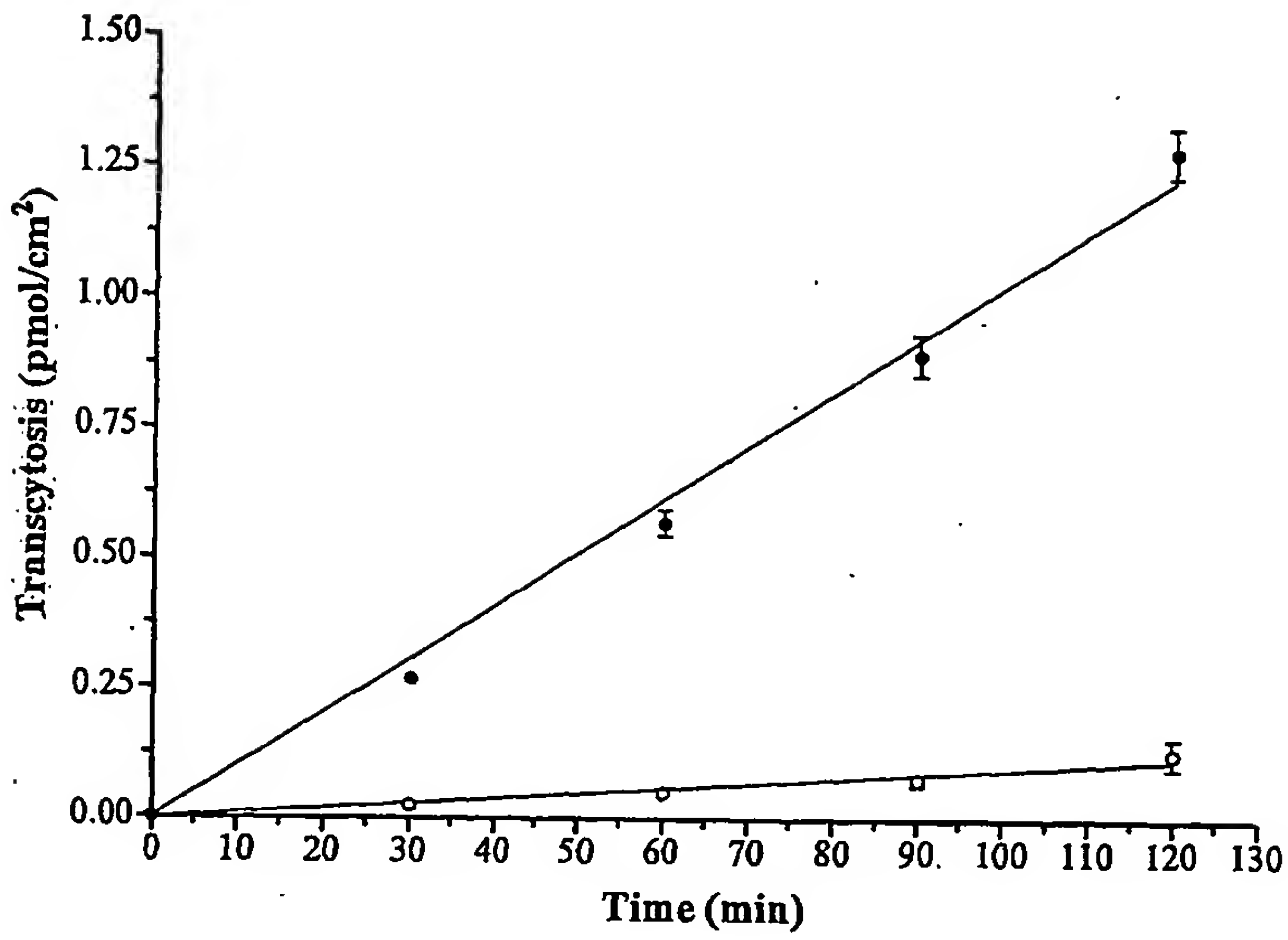
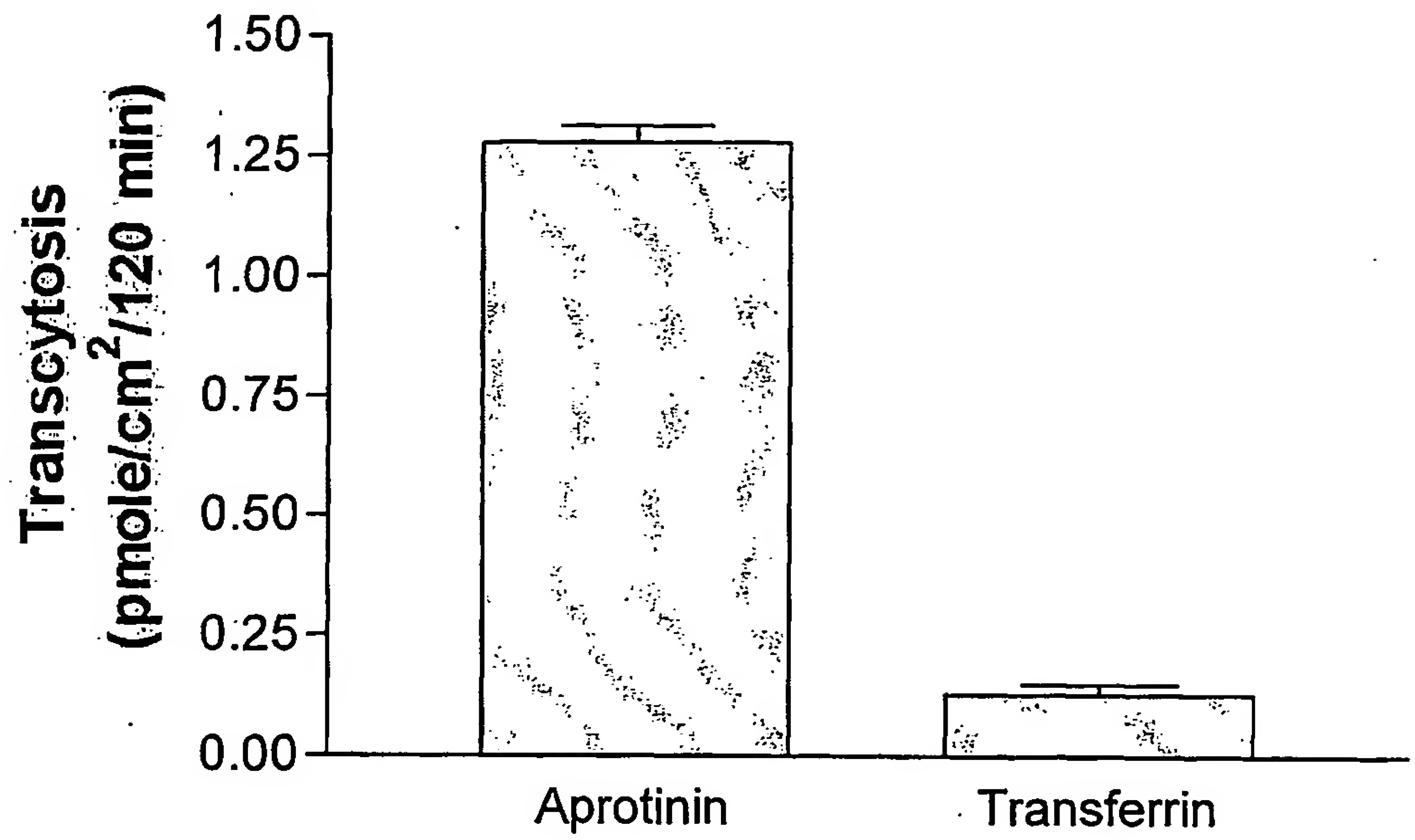

1.50
1.25
1.00
0.75
0.50
0.25
0.00
Transcytosis (pmol/cm²)
0 10 20 30 40 50 60 70 80 90 100 110 120 130
Time (min)

FIG. 2



Transcytosis (pmole/cm^2/120 min)
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Aprotinin
Transferrin

FIG. 3

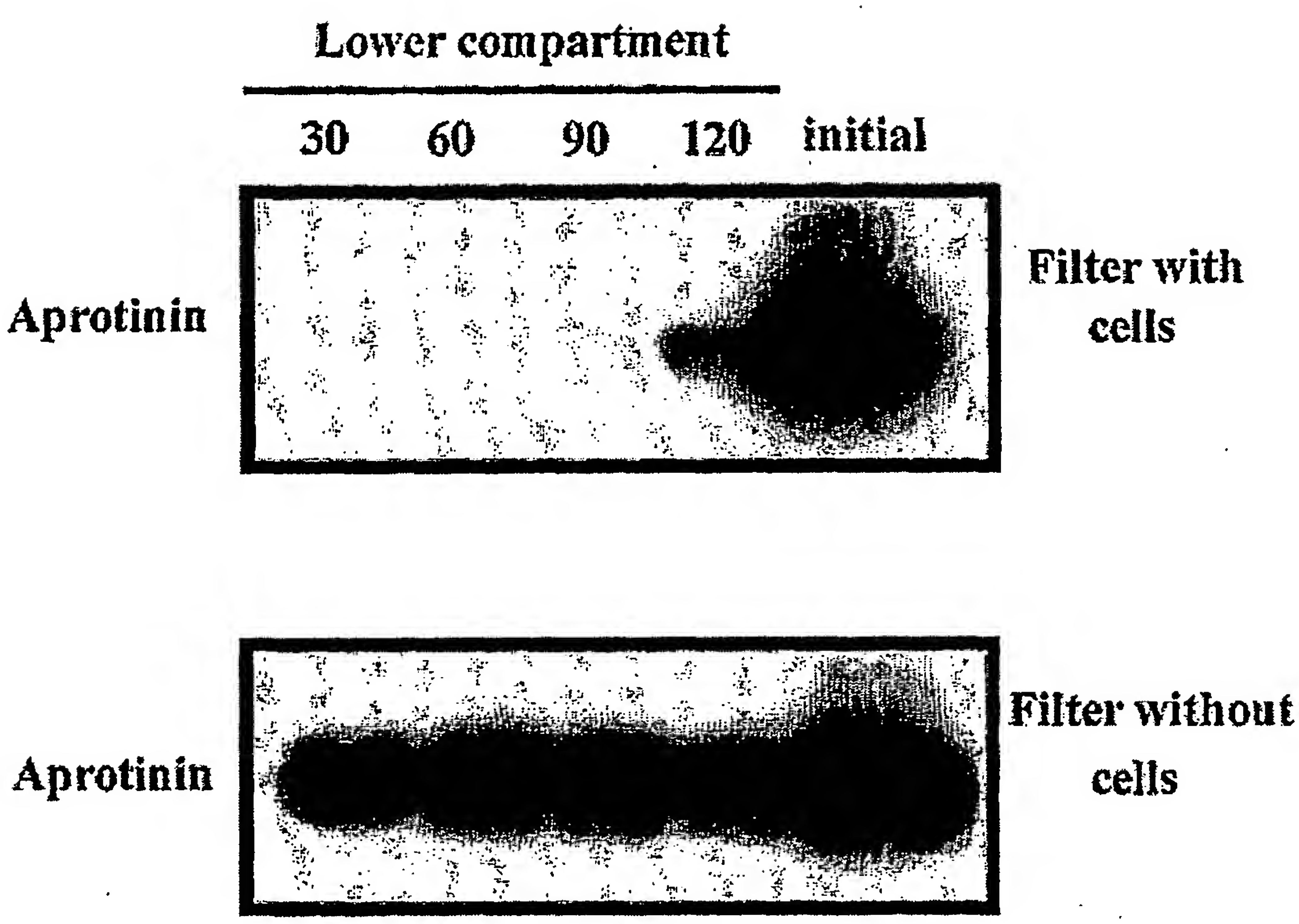
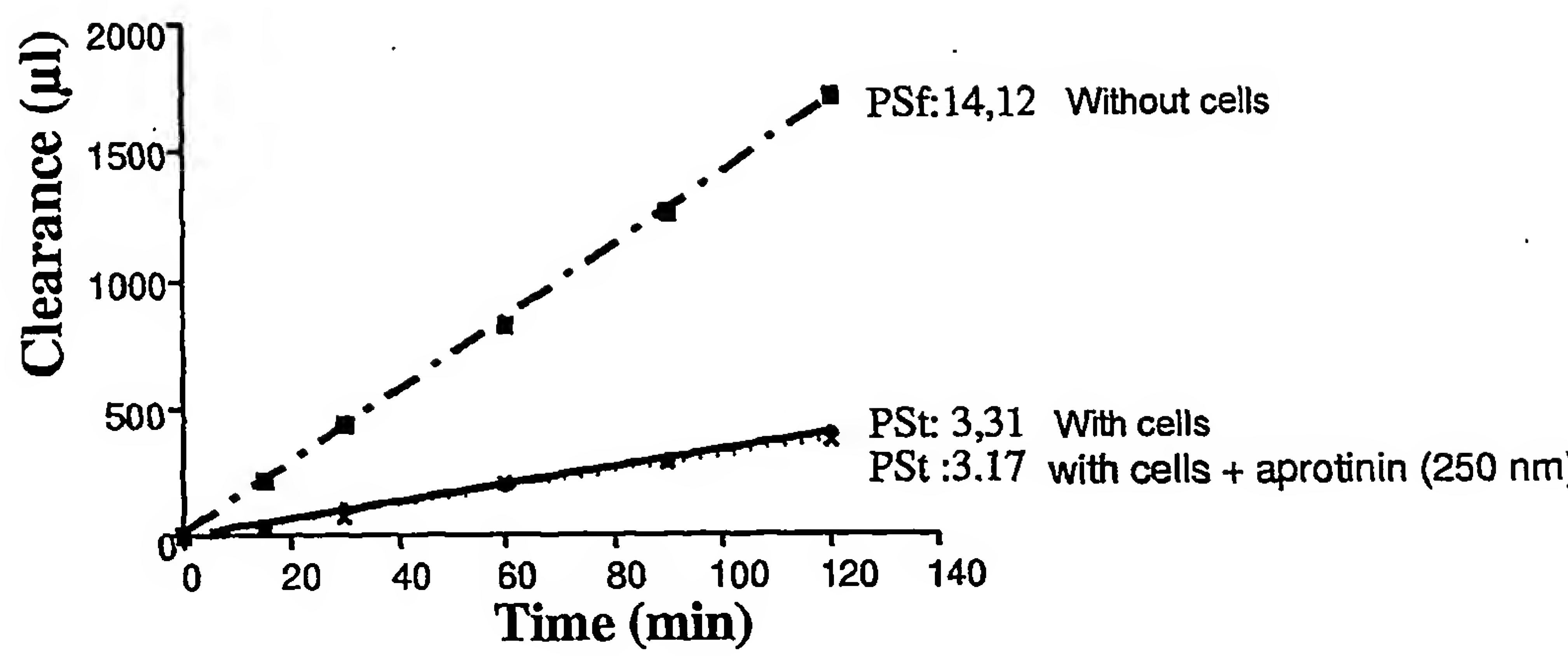
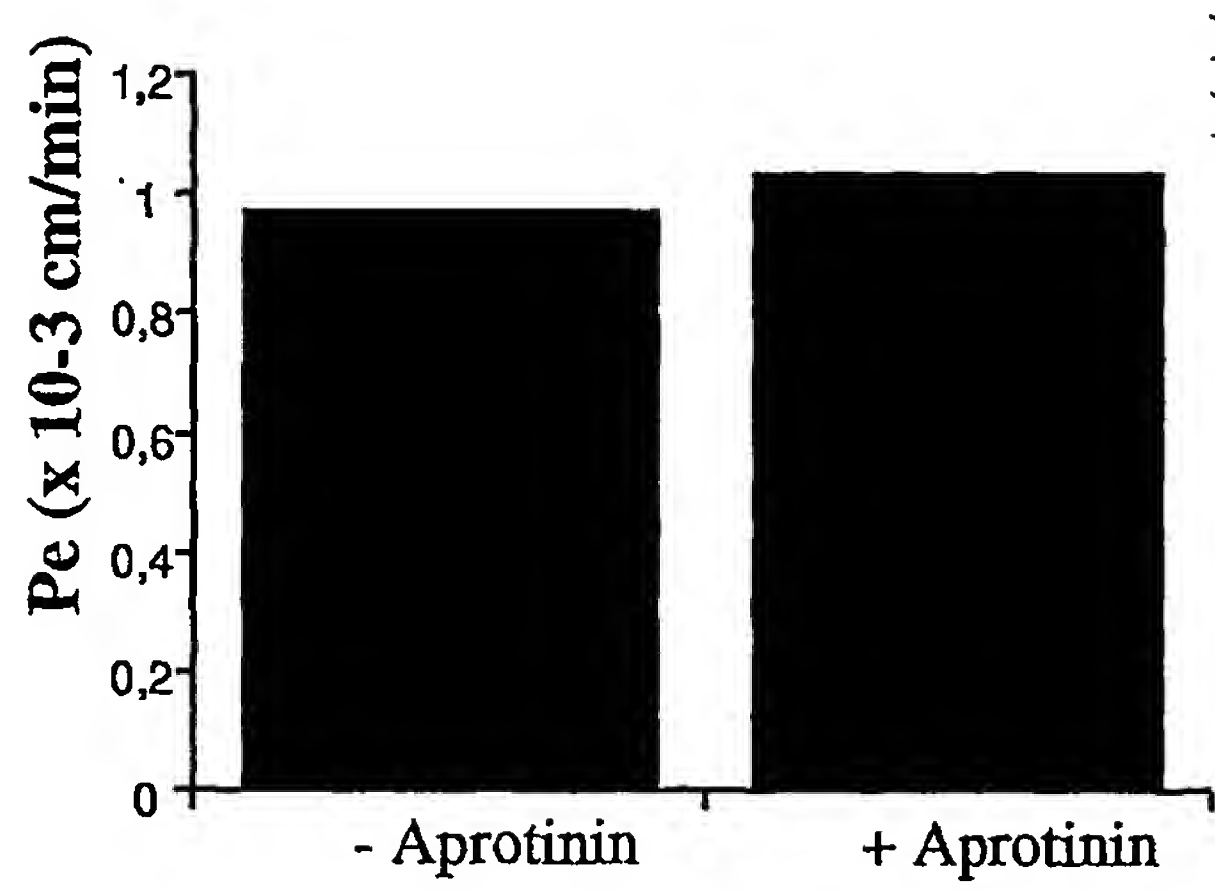

Lower compartment
30
60
90
120
initial
Aprotinin
Filter with cells
Aprotinin
Filter without cells

FIG. 4



Clearance (µl)
2000
1500
1000
500
0
0
20
40
60
80
100
120
140
Time (min)
PSf:14,12 Without cells
PSt: 3,31 With cells
PSt :3.17 with cells + aprotinin (250 nm)

FIG. 5



Pe without aprotinin: 0.97
Pe with aprotinin: 1.03

FIG. 6

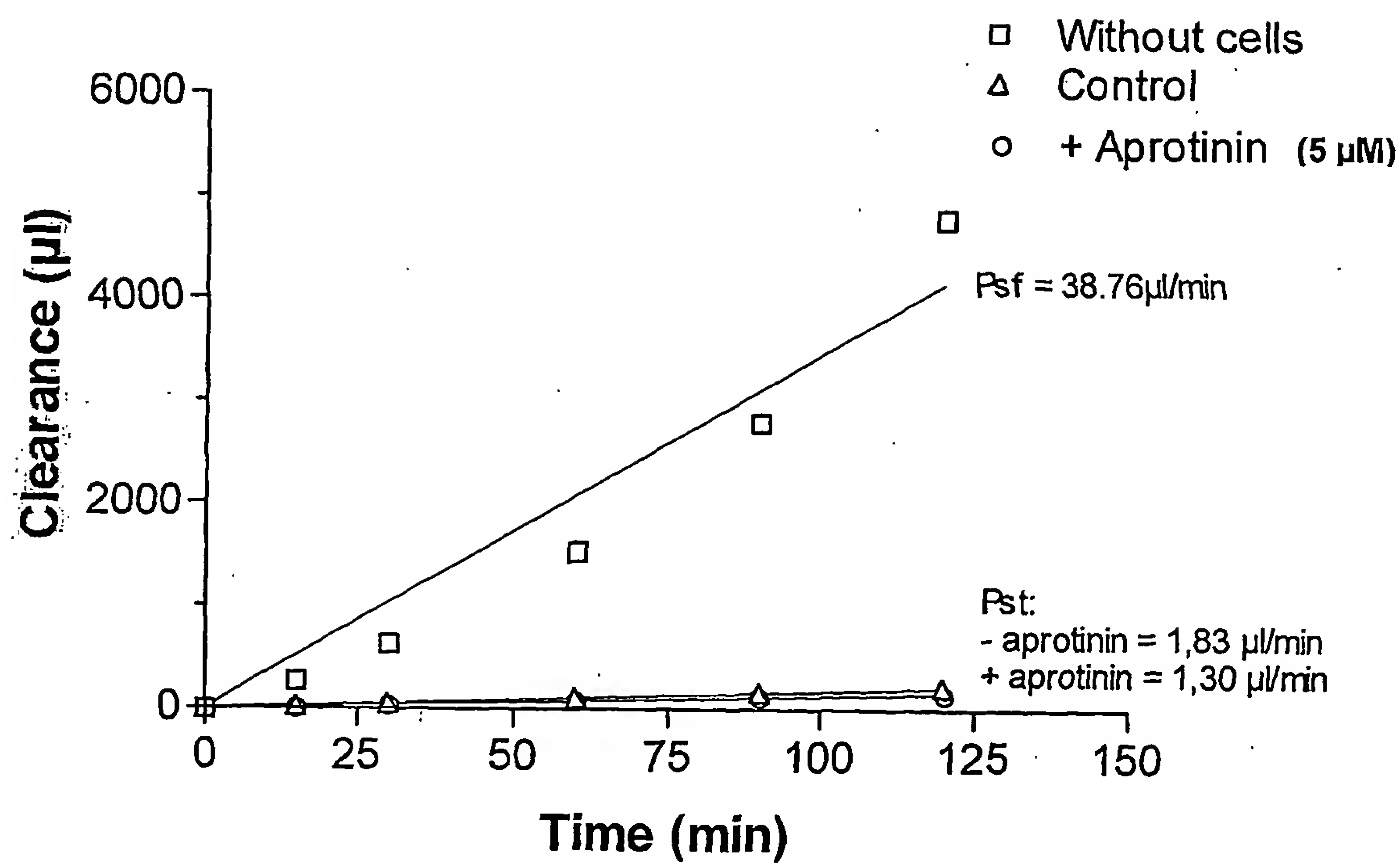

Without cells
Control
+ Aprotinin (5 µM)
6000
4000
2000
0
Clearance (µl)
Psf = 38.76µl/min
Pst:
- aprotinin = 1,83 µl/min
+ aprotinin = 1,30 µl/min
0
25
50
75
100
125
150
Time (min)

FIG. 7

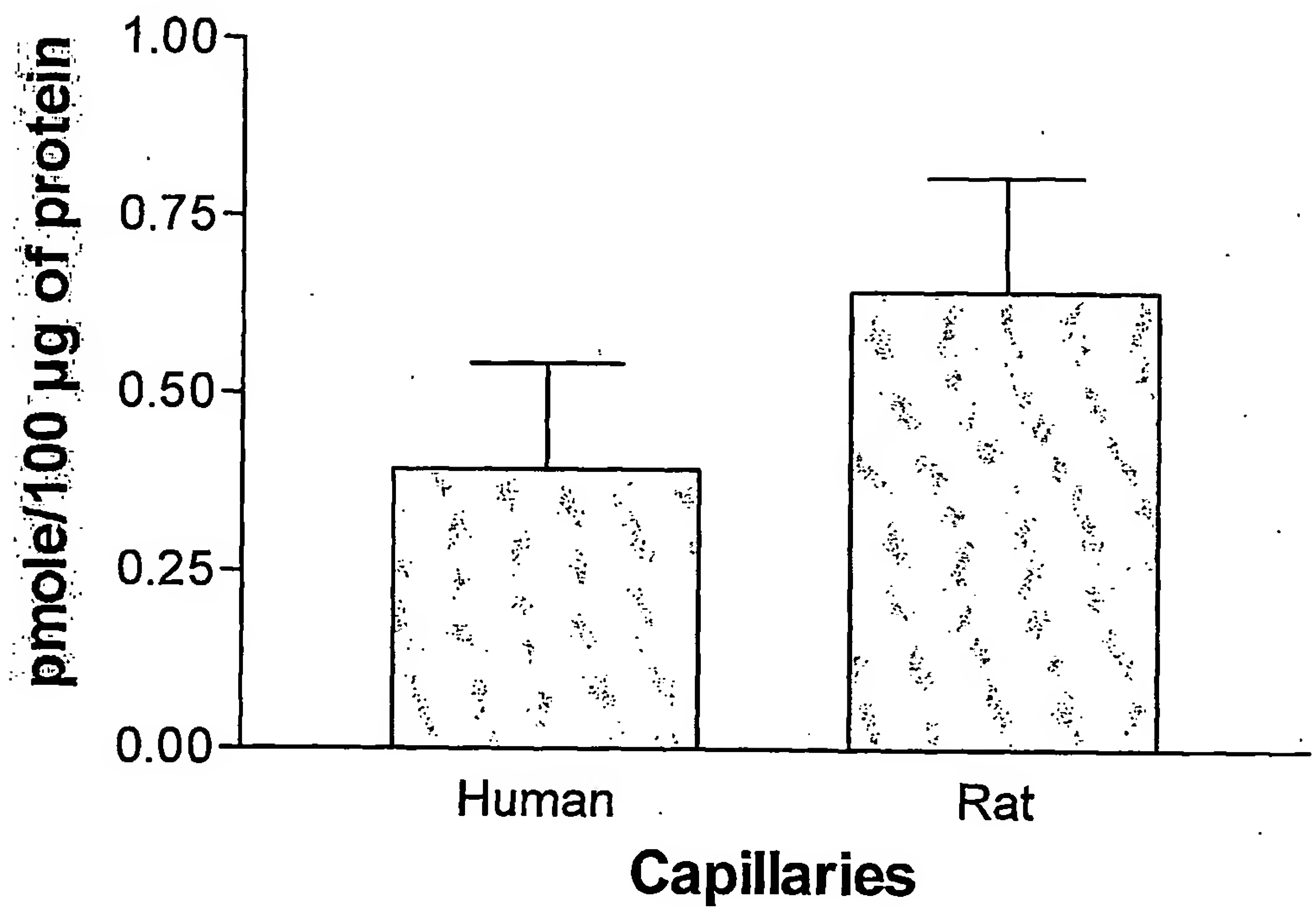

1.00
0.75
0.50
0.25
0.00
pmole/100 µg of protein
Human
Rat
Capillaries

FIG. 8

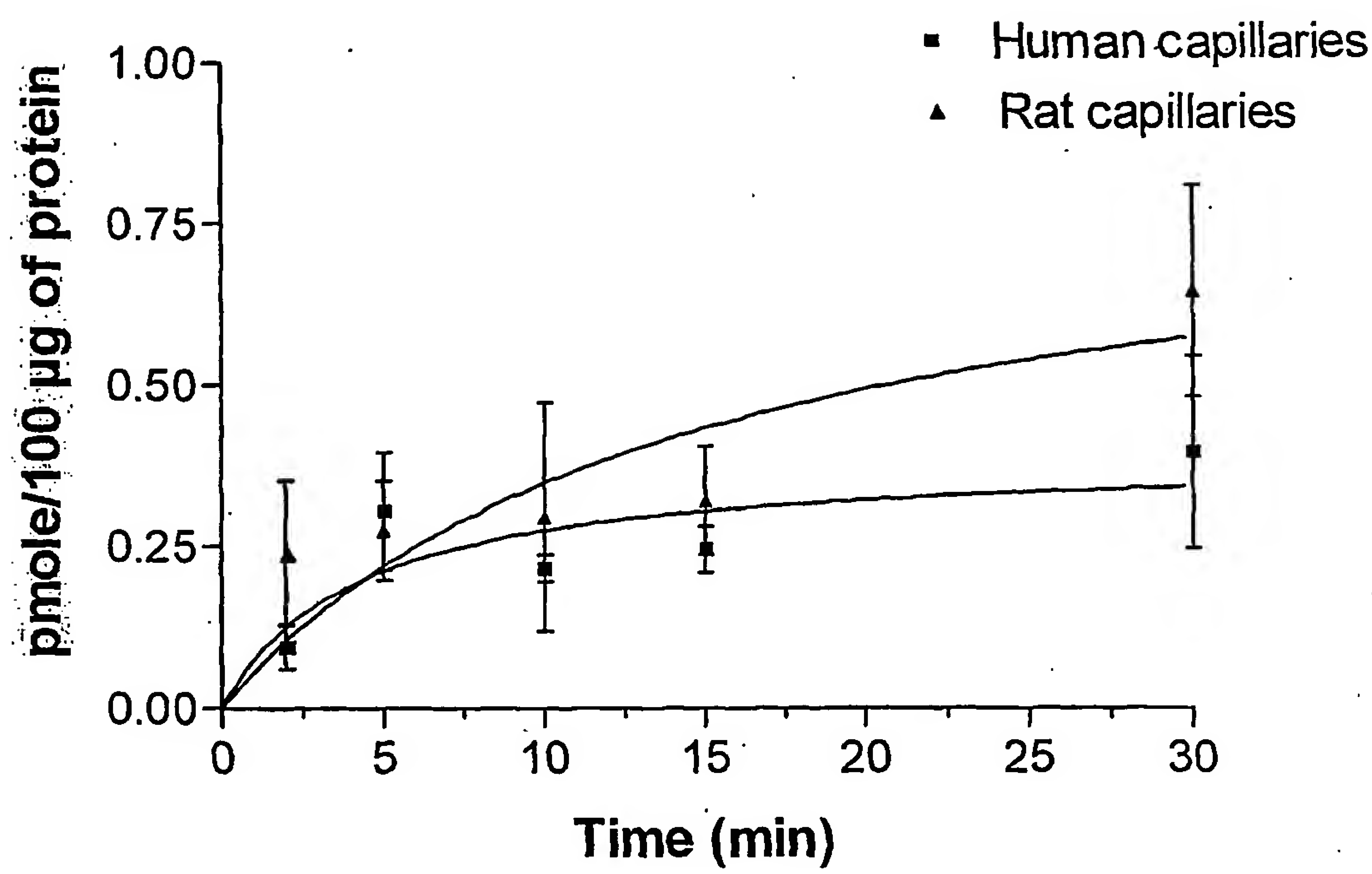

Human capillaries
Rat capillaries
pmole/100 µg of protein
1.00
0.75
0.50
0.25
0.00
0
5
10
15
20
25
30
Time (min)

FIG. 9

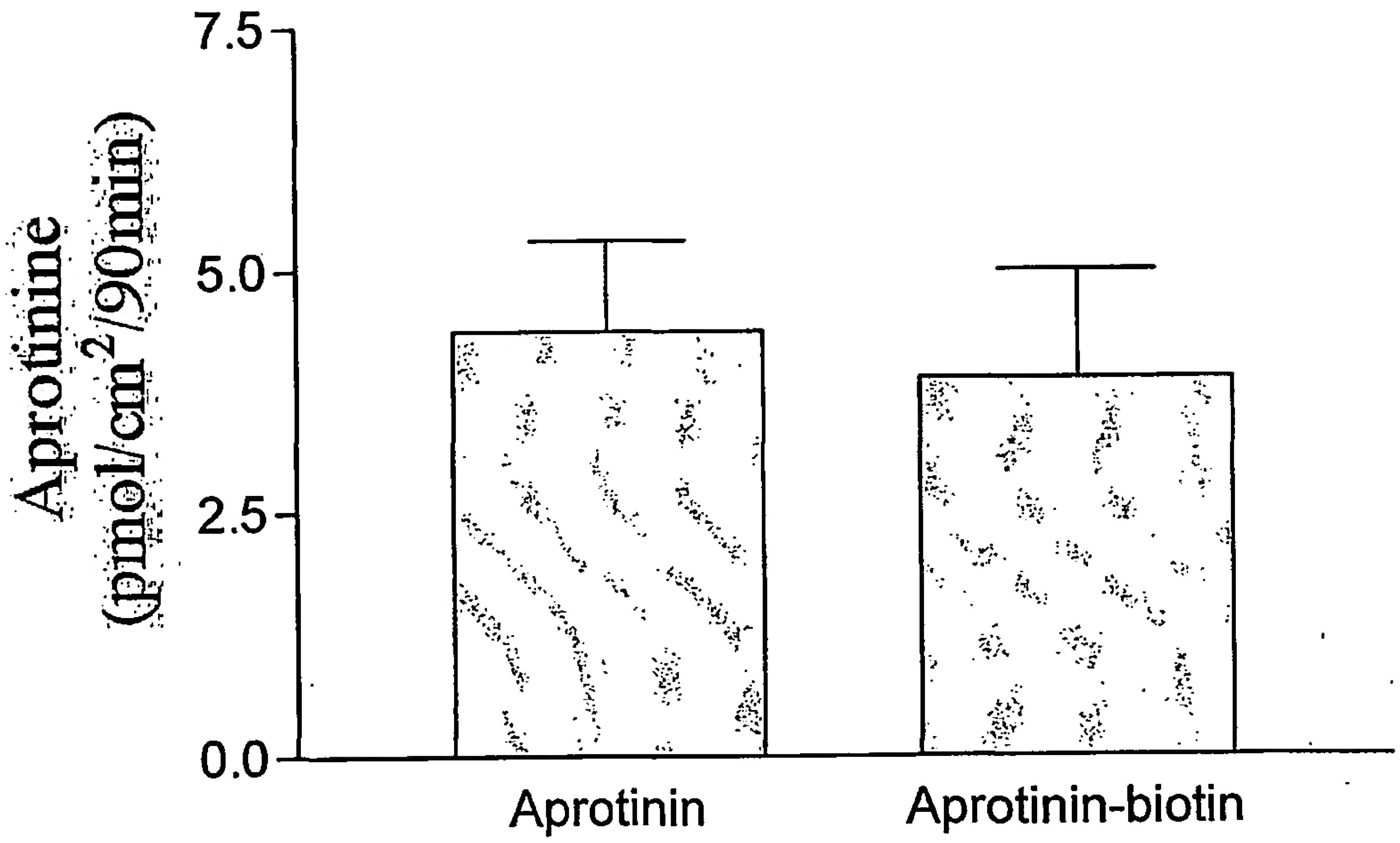

7.5
5.0
2.5
0.0
Aprotinine
(pmol/cm²/90min)
Aprotinin
Aprotinin-biotin

FIG. 10

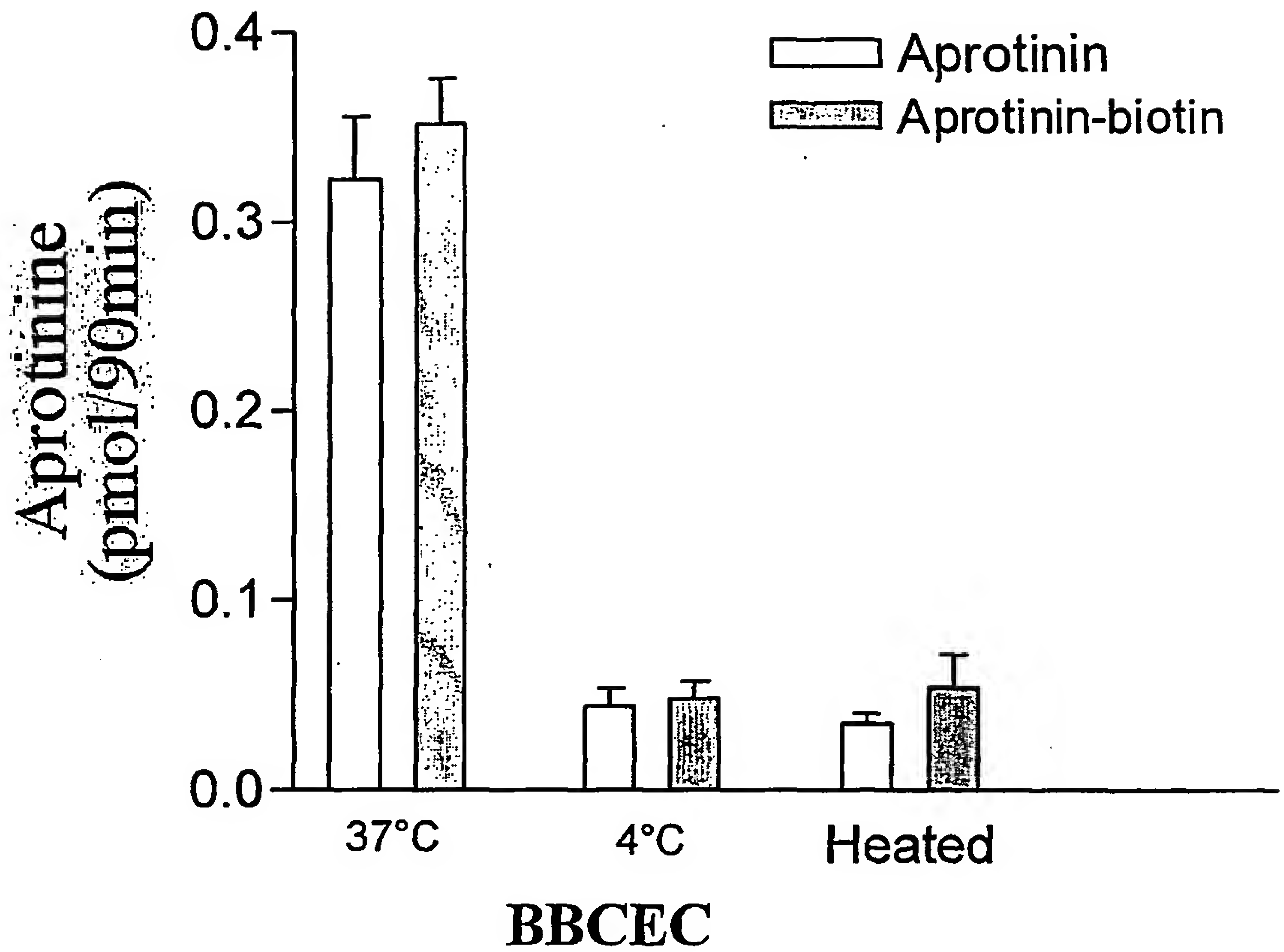

0.4
0.3
0.2
0.1
0.0
Aprotinine (pmol/90min)
Aprotinin
Aprotinin-biotin
37°C
4°C
Heated
BBCEC

FIG. 11

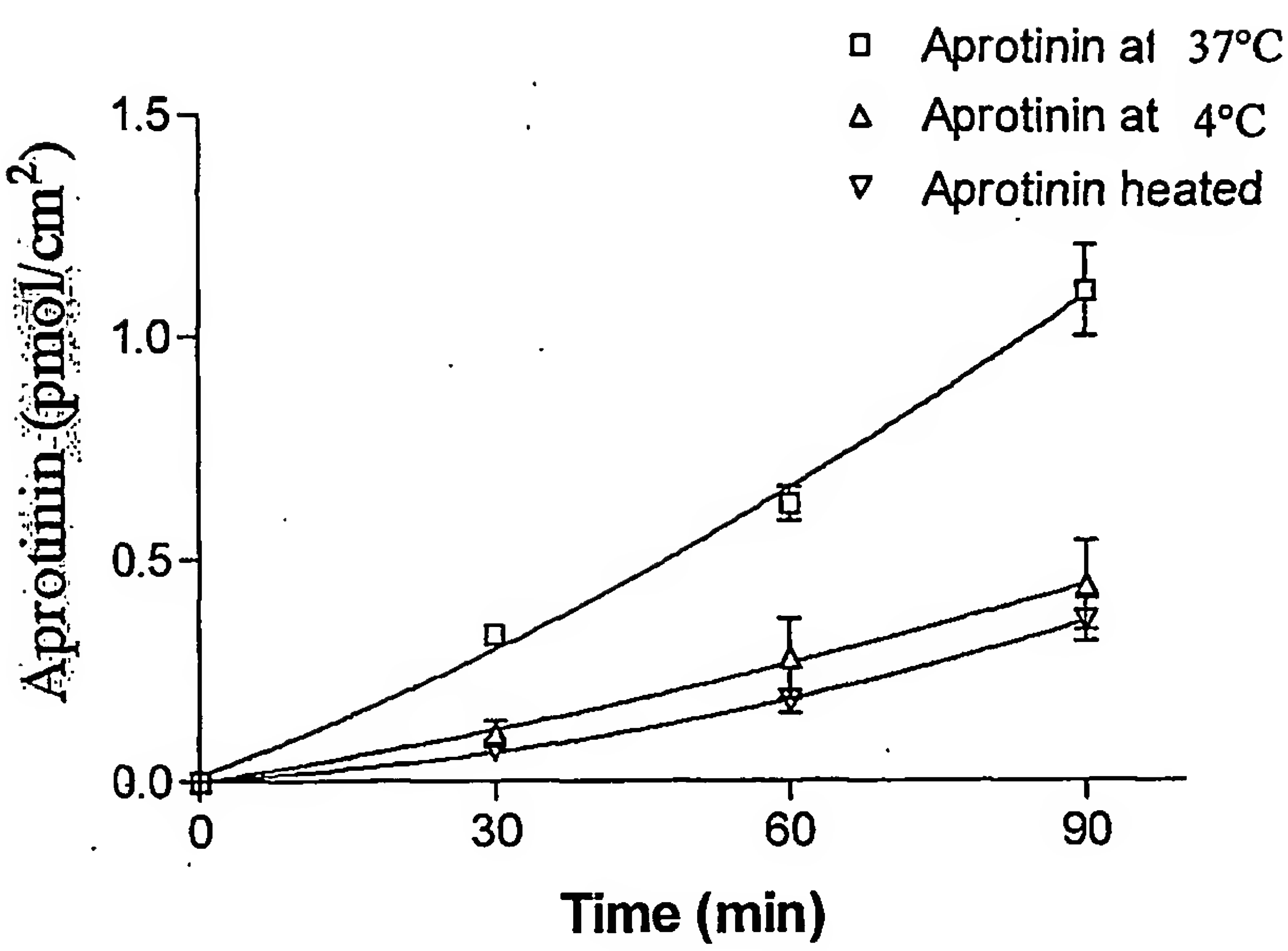


Fig. 12A

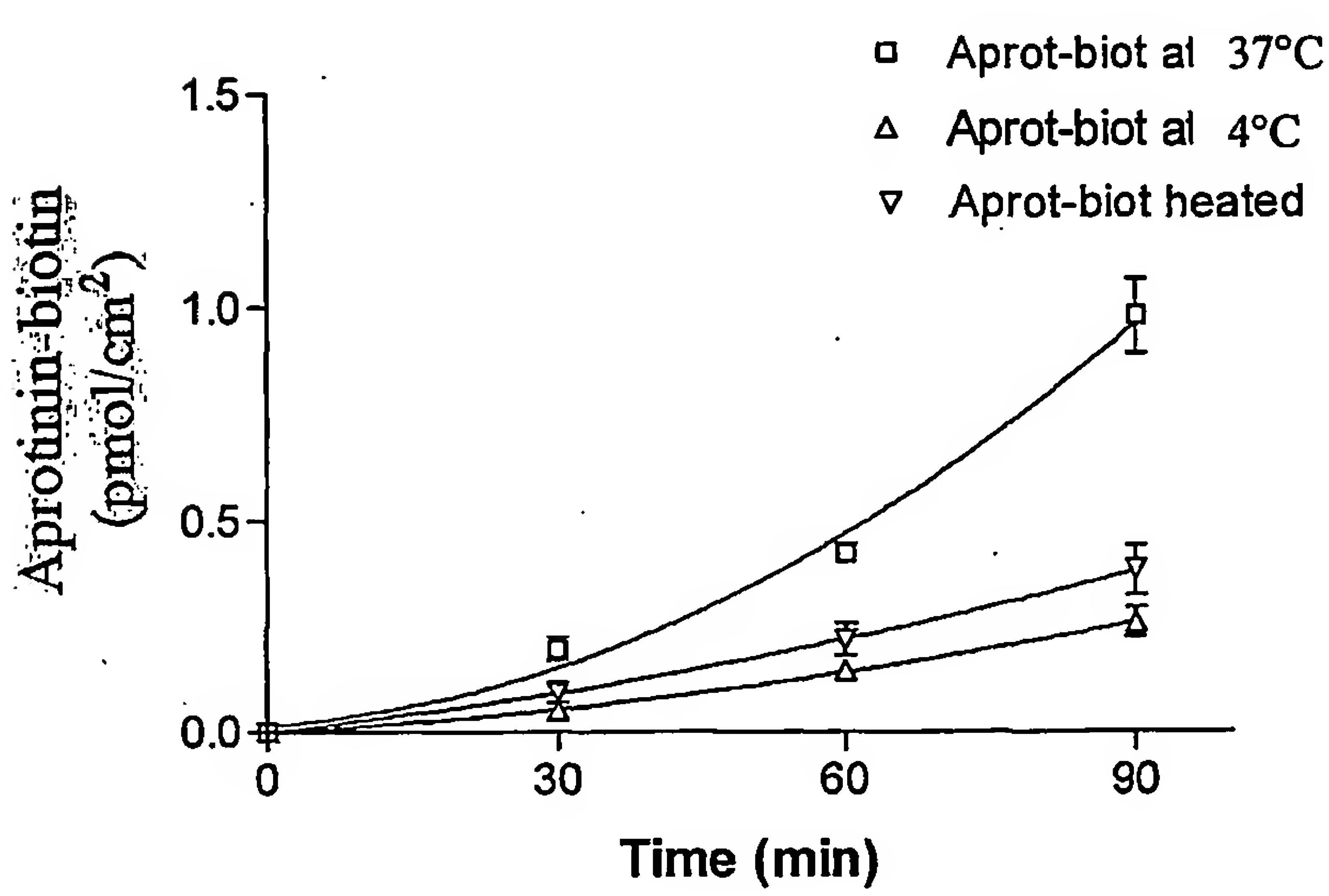


FIG. 12B

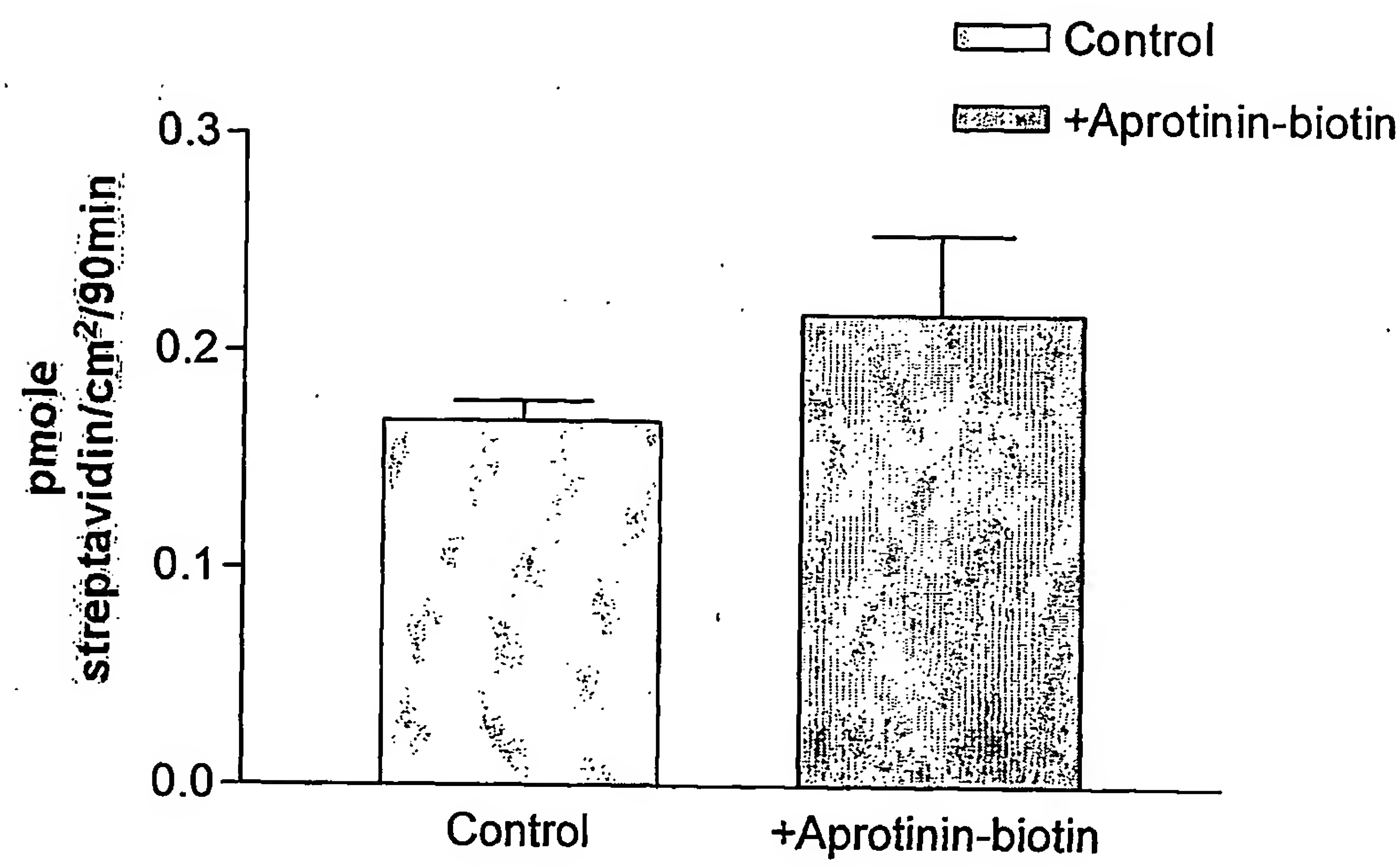

Control
+Aprotinin-biotin
0.3
0.2
0.1
0.0
pmole streptavidin/cm²/90min
Control
+Aprotinin-biotin

FIG. 13

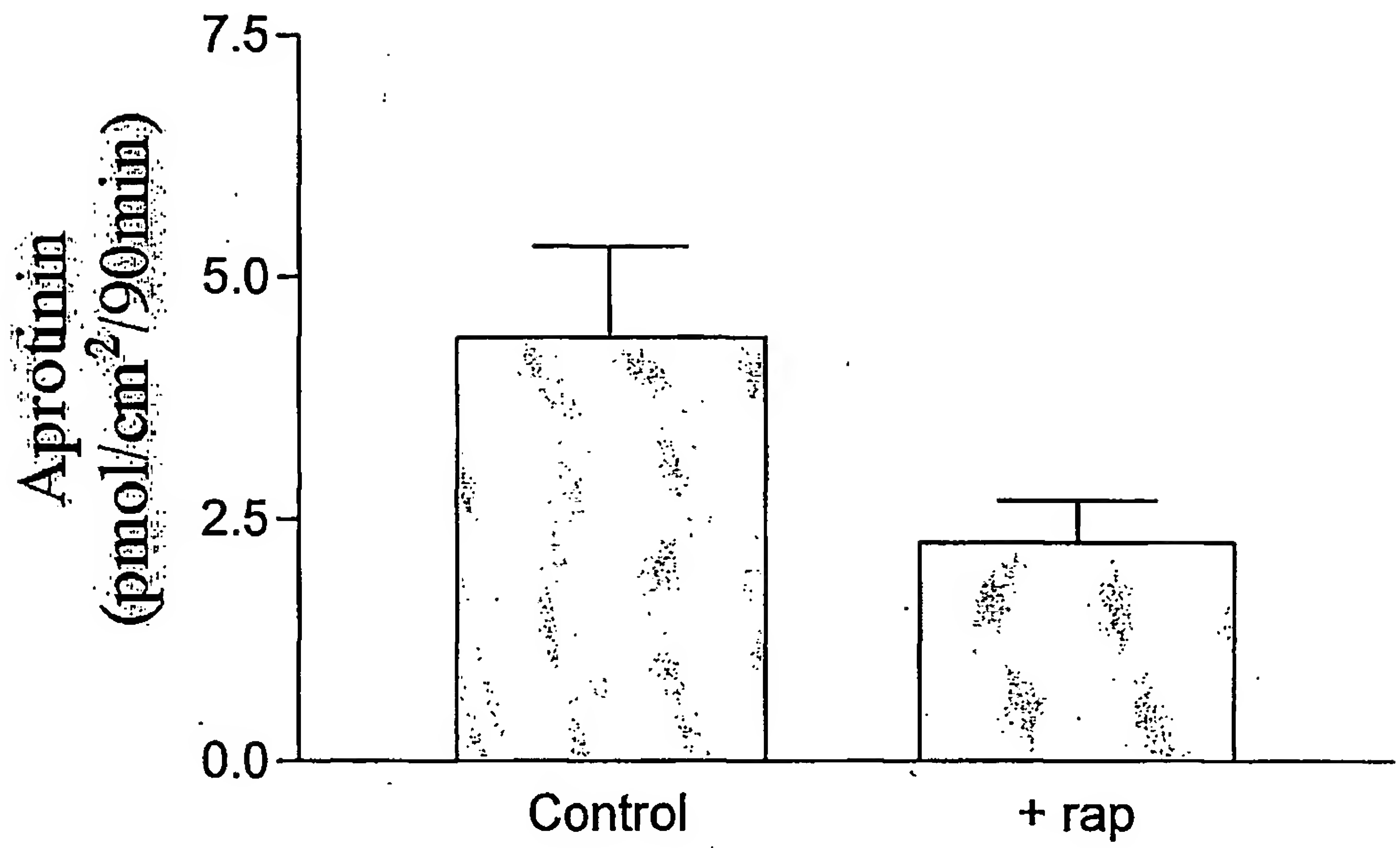
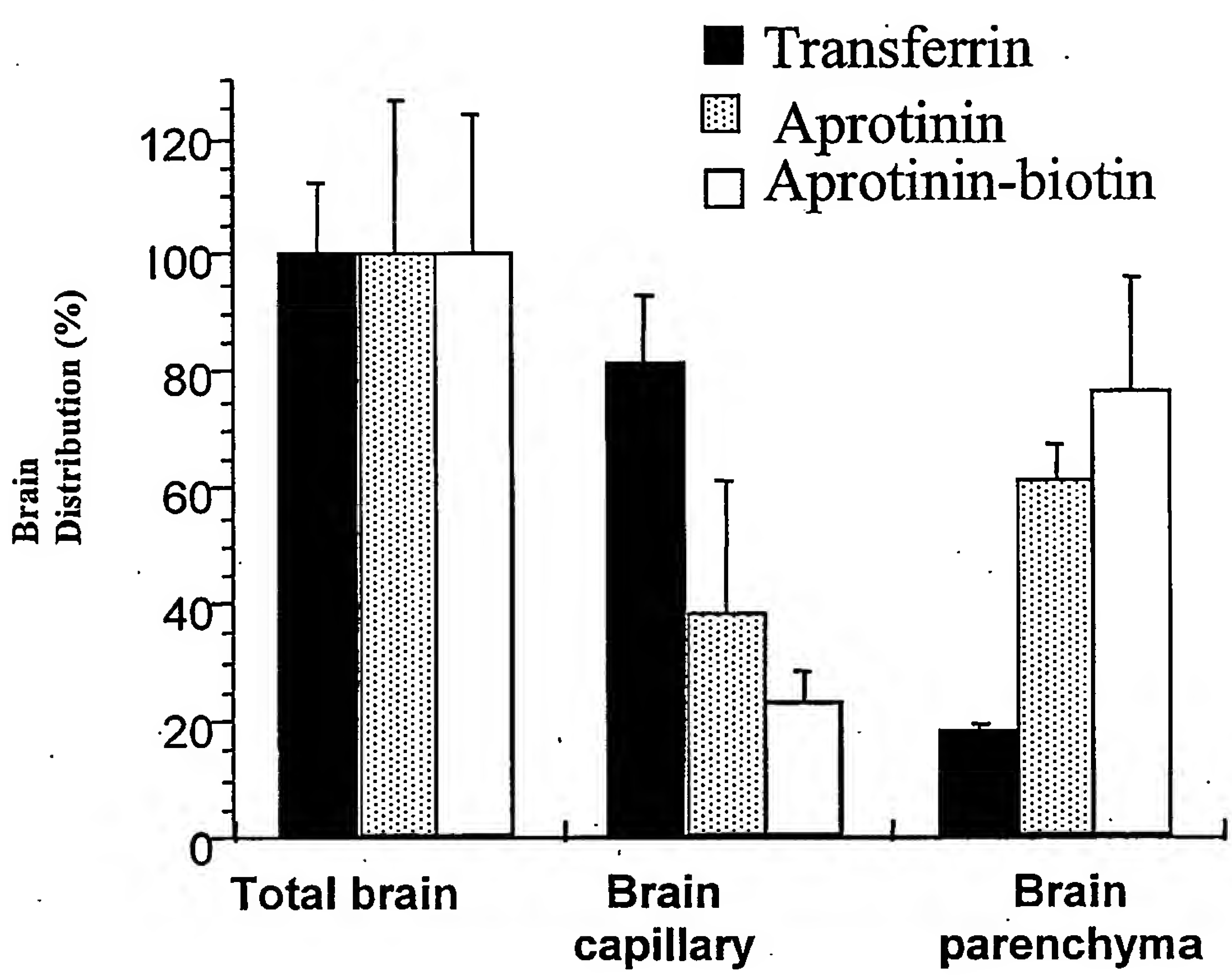
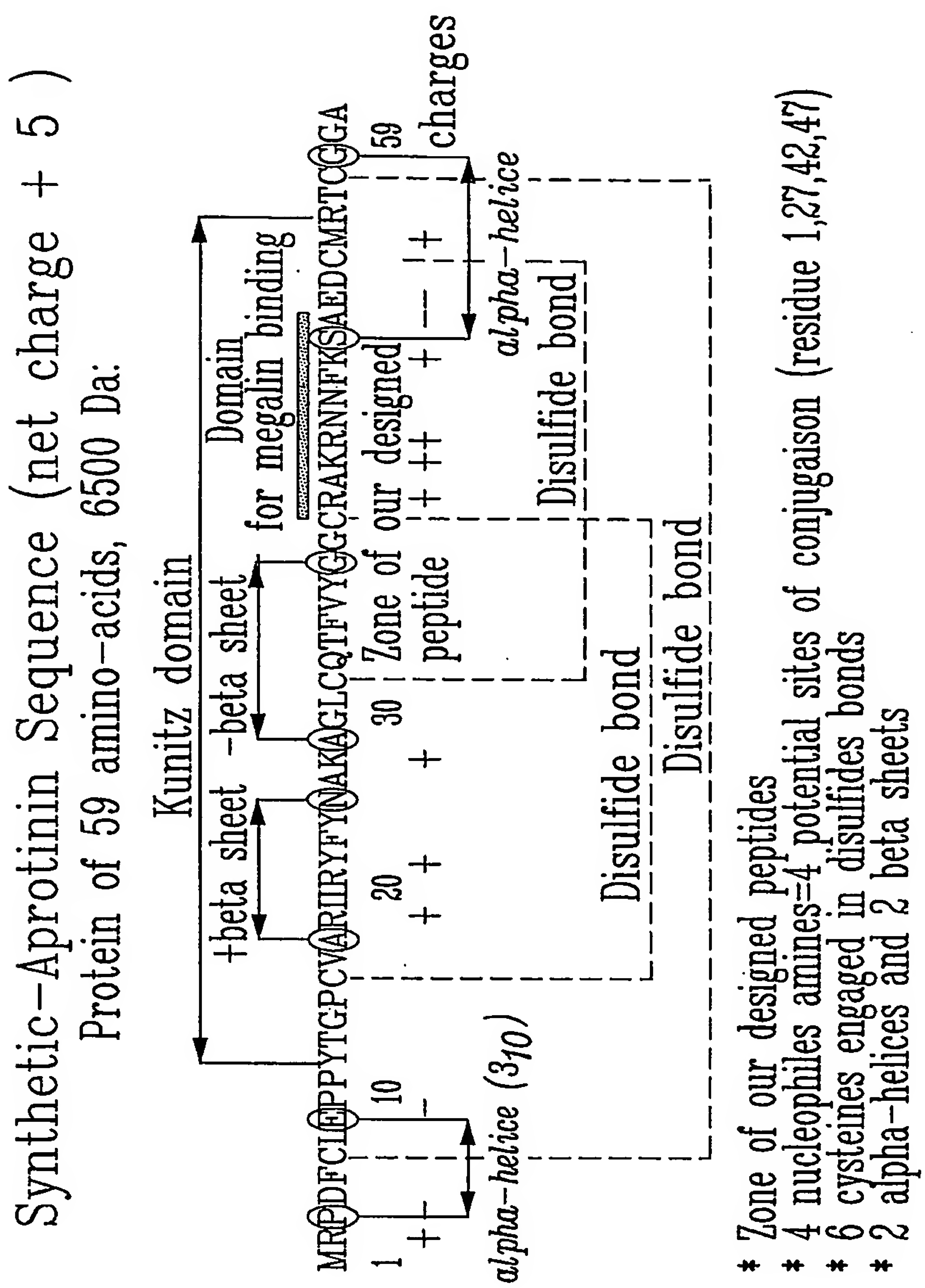


FIG. 14



V_D for aprotinin in brain parenchyma = 3 µl/100 g

FIG. 15

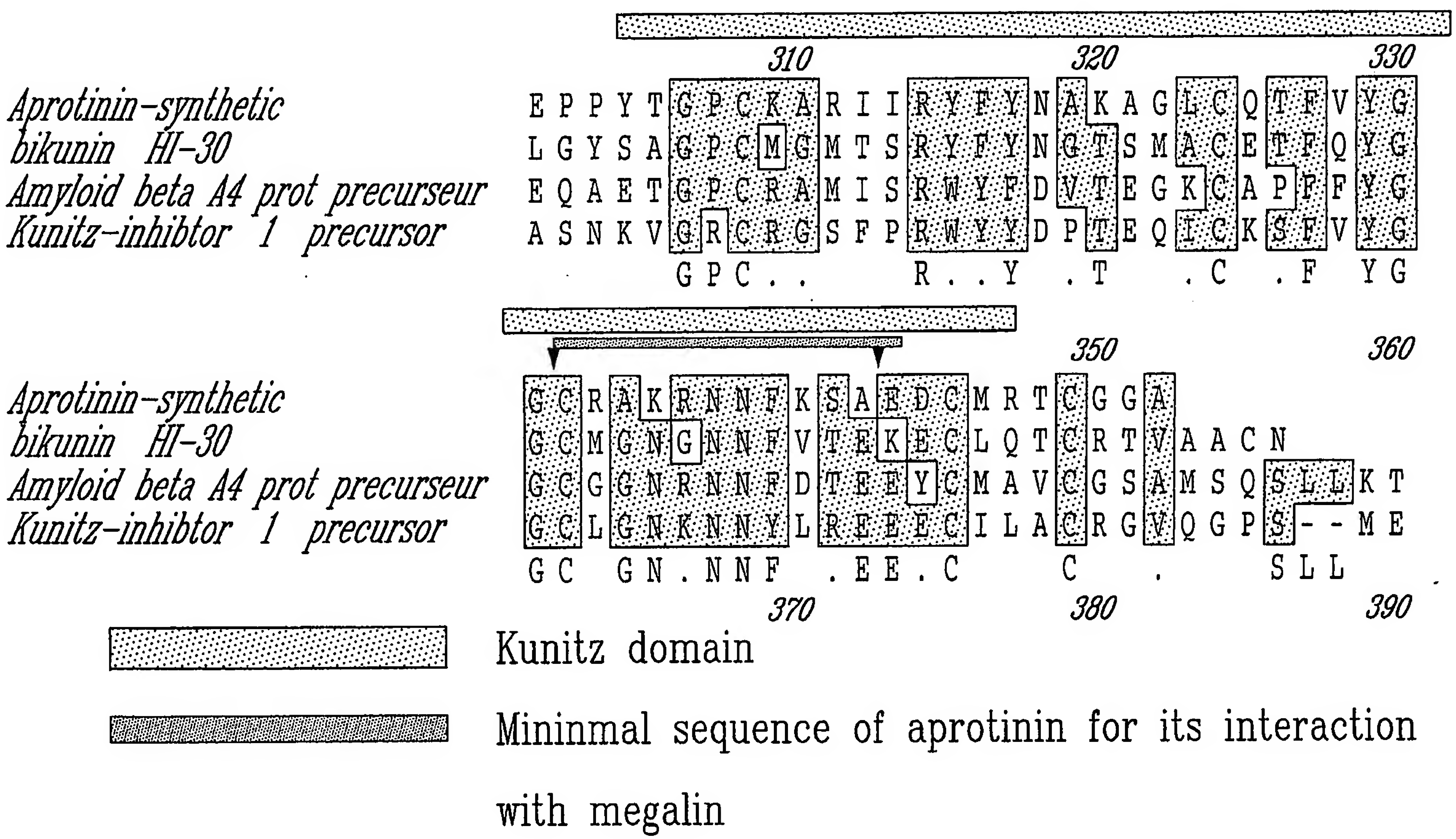


Synthetic-Aprotinin Sequence (net charge + 5)
Protein of 59 amino-acids, 6500 Da:
Kunitz domain
+beta sheet
-beta sheet
Domain for megalin binding
MRPDFCLEPPYTGPCVARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA
1
10
20
30
59
charges
Zone of our designed peptide
alpha-helice (3_{10})
alpha-helice
Disulfide bond
Disulfide bond
Disulfide bond
* Zone of our designed peptides
* 4 nucleophiles amines=4 potential sites of conjugaison (residue 1,27,42,47)
* 6 cysteines engaged in disulfides bonds
* 2 alpha-helices and 2 beta sheets

FIG. 16

FIG. 17

Alignement between aprotinin and three human proteins with a similar domain



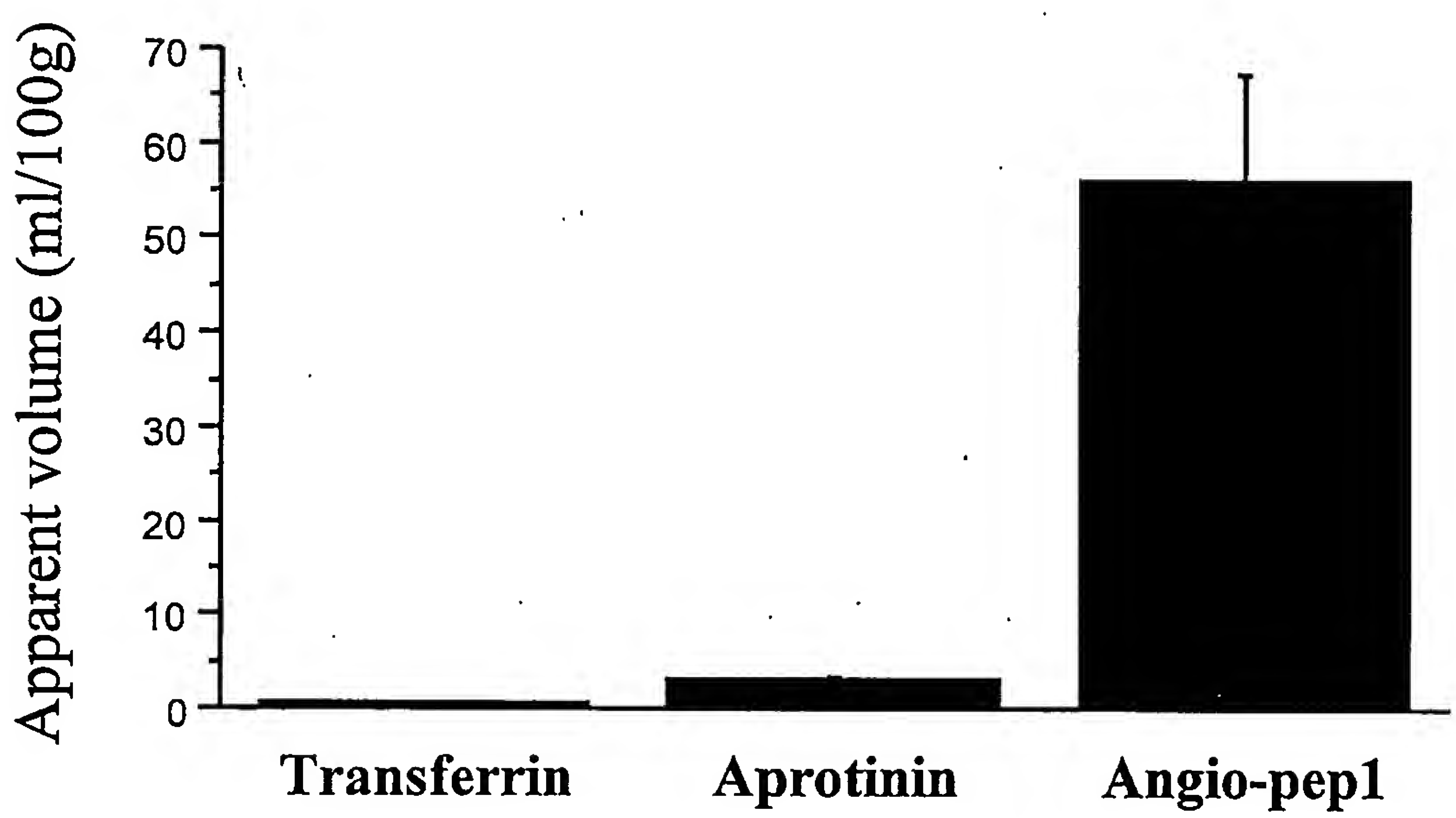

Apparent volume (ml/100g)
70
60
50
40
30
20
10
0
Transferrin
Aprotinin
Angio-pep1

FIG. 18

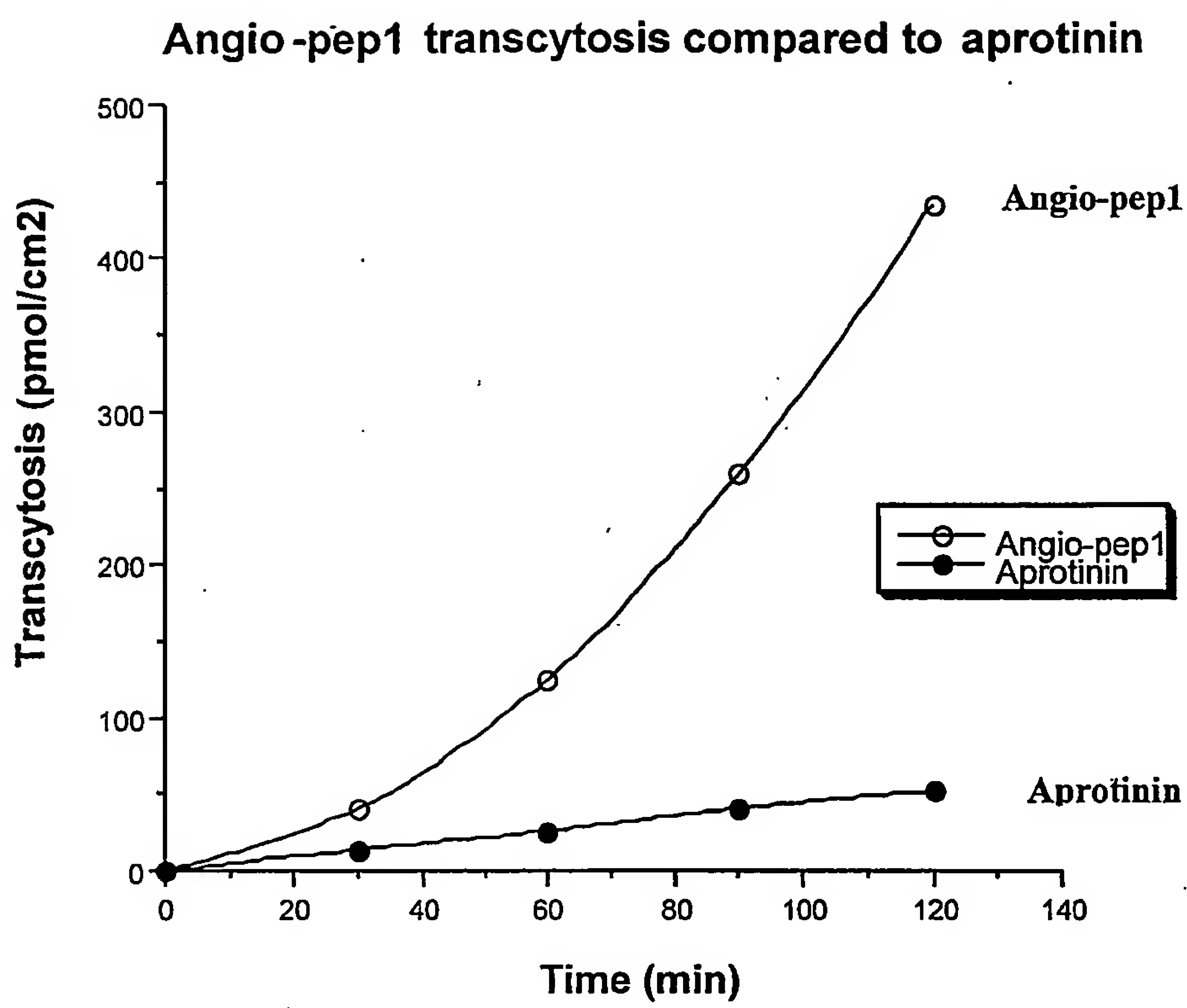

Angio-pep1 transcytosis compared to aprotinin
Transcytosis (pmol/cm2)
500
400
300
200
100
0
0
20
40
60
80
100
120
140
Time (min)
Angio-pep1
Aprotinin
Angio-pep1
Aprotinin

FIG. 19

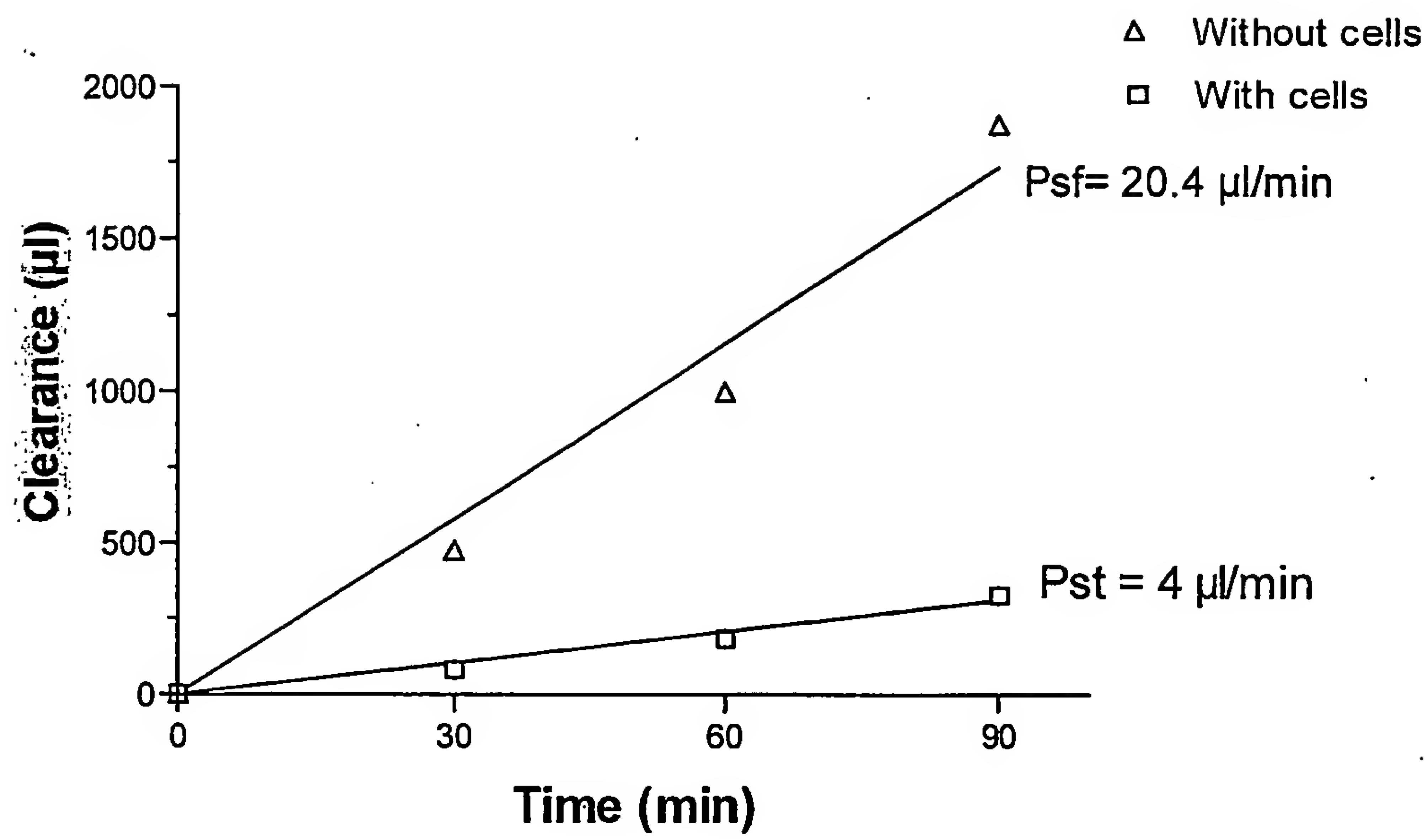

Without cells
With cells
Psf= 20.4 µl/min
Pst = 4 µl/min
Clearance (µl)
2000
1500
1000
500
0
0
30
60
90
Time (min)

FIG. 20

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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60/437,986 6 January 2003 (06.01.2003) US

(71) Applicant *(for all designated States except US)*: **TRANSFERT PLUS** [CA/CA]; 550 Sherbrooke West, Suite 100, H3A 1B9 Montréal, Québec (CA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BÉLIVEAU, Richard** [CA/CA]; 266 Wilson, H3E 1L8 Montréal, Québec, CA (CA). **DEMEULE, Michel** [CA/CA]; 3557 Archambault, J4M 2W8 Longueuil, Québec, CA (CA).

(74) Agent: **OGILVY RENAULT**; Suite 1600, 1981 McGill College Avenue, H3A 2Y3 Montréal, Québec, CA (CA).

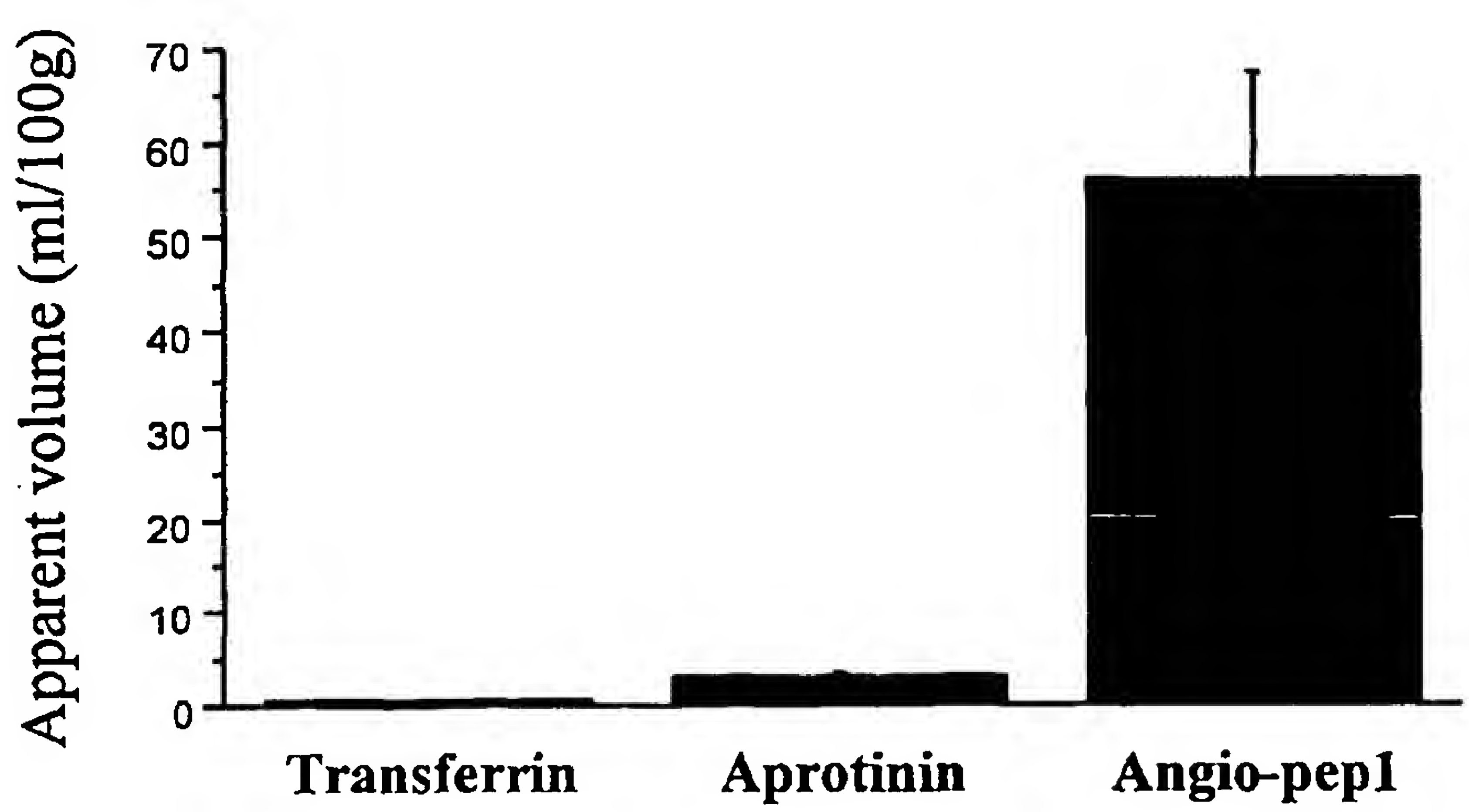
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

[Continued on next page]

(54) Title: APROTININ AND ANGLOS AS CARRIERS ACROSS THE BLOOD-BRAIN BARRIER



(57) Abstract: The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier. The present invention relates to improvements in the field of drug delivery. More particularly, the invention relates to a non-invasive and flexible method and carrier for transporting a compound or drug across the blood-brain barrier of an individual. In particular the present invention relates to a carrier for transporting an agent attached thereto across a blood-brain barrier, wherein the carrier is able to cross the blood-brain barrier after attachment to the agent and thereby transport the agent across the blood-brain barrier.

WO 2004/060403 A3

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report: 18 November 2004

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA2004/000011

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 A61P25/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, CHEM ABS Data, EMBASE, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 199 53 696 A (CHERKASKY ALEXANDER) 10 May 2001 (2001-05-10) column 1, lines 15-32; claim 3; figure 1 ----- -/--	1-4, 7-21, 24-38, 41-53, 56-67, 70-78, 80-102

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
19 May 2004	30. 09. 2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gonzalez Ramon, N

5

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHIMURA T ET AL: "TRANSPORT MECHANISM OF A NEW BEHAVIORALLY HIGHLY POTENT ADRENOCORTICOTROPIC HORMONE (ACTH) ANALOG, EBIRATIDE, THROUGH THE BLOOD-BRAIN BARRIER" JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS, AMERICAN SOCIETY FOR PHARMACOLOGY AND, US, vol. 258, no. 2, 1991, pages 459-465, XP008030272 ISSN: 0022-3565 abstract; figure 1 -----	1,2,4, 7-19,21, 24-36, 38, 41-51, 53, 56-65, 67, 70-77, 80-102
Y	DEMEULE M ET AL: "HIGH TRANSCYTOSIS OF MELANOTRANSFERRIN (P97) ACROSS THE BLOOD-BRAIN BARRIER" JOURNAL OF NEUROCHEMISTRY, NEW YORK, NY, US, vol. 83, no. 4, November 2002 (2002-11), pages 924-933, XP001188983 ISSN: 0022-3042 see discussion abstract -----	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102
Y	SEIDEL G ET AL: "EFFECTS OF TRASYLOL ON THE BLOOD-BRAIN BARRIER IN RATS" NAUNYN-SCHMIEDEBERG'S ARCHIVES OF PHARMACOLOGY, SPRINGER, BERLIN, DE, vol. 284, no. 4, 1974, page R73, XP008030270 ISSN: 0028-1298 abstract -----	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102
Y	MARTEL ET AL: "Transport of apolipoproteins E and J at the blood - brain barrier. Relevance to Alzheimer's disease" STP PHARMA SCIENCES, PARIS, FR, vol. 7, no. 1, 1997, pages 28-36, XP002090769 ISSN: 1157-1489 abstract -----	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102
P,X	WO 03/009815 A (KENNARD MALCOLM L ;YANG JOSEPH (CA); DEMEULE MICHEL (CA); BELIVEAU) 6 February 2003 (2003-02-06) page 4; figure 17 page 37, line 8; claims 8,25 -----	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102

-/--

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA2004/000011

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/33090 A (PROCYON BIOPHARMA INC) 25 April 2002 (2002-04-25) page 1, lines 42,43; claims 12,22,36,46,52,58,75,82,88; example 18	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102
A	GUILLOT F L ET AL: "ANGIOTENSIN PEPTIDE REGULATION OF BOVINE BRAIN MICROVESSEL ENDOTHELIAL CELL MONOLAYER PERMEABILITY" JOURNAL OF CARDIOVASCULAR PHARMACOLOGY, NEW YORK, NY, US, vol. 18, no. 2, 1991, pages 212-218, XP008030278 ISSN: 0160-2446 abstract page 217, column 2	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102
P,Y	KOBAYASHI H ET AL: "THE PROTEASE INHIBITOR BIKUNIN, A NOVEL ANTI-METASTATIC AGENT" BIOLOGICAL CHEMISTRY, XX, XX, vol. 384, no. 5, 1 May 2003 (2003-05-01), pages 749-754, XP008030275 ISSN: 1431-6730 abstract; figure 2	1-5, 7-22, 24-39, 41-54, 56-68, 70-78, 80-102

5

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2004/000011

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4, 7-21, 24-38, 41-53, 56-67, 70-78, 80-102 (all partially), 5, 22, 39
54, 68

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 5, 22, 39, 54, 68 complete; 1-4, 7-21, 24-38, 41-53, 56-67, 70-78, 80-102 partially

   Carrier for transporting an agent atached thereto across the blood brain barrier wherein the agent is anticancer agent paclitaxel. Conjugate comprising the carrier and paclitaxel, pharmaceutical composition and use of the same for neurological disease (brain tumour, brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and obesity).

   ---

2. claims: 1-4, 6-21, 23-38, 40-53, 55-67, 69-79, 80-102 partially

   Carrier for transporting an agent atached thereto across the blood brain barrier wherein the agent is a green fluorescent protein, a histag protein, and beta galactosidase. Conjugate comprising the carrier and the protein agent, pharmaceutical composition and use of the same for neurological disease (brain tumour, brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and obesity)

   ---

3. claims: 1-4, 6-21, 23-38, 40-53, 55-67, 69-79, 80-102 partially

   Carrier for transporting an agent atached thereto across the blood brain barrier wherein the agent is a green fluorescent protein, a histag protein, and beta galactosidase. Conjugate comprising the carrier and the protein agent, pharmaceutical composition and use of the same for neurological disease (brain tumour, brain metastasis, schizophrenia, epilepsy, Alzheimer's disease, Parkinson's disease, Huntington's disease, stroke and obesity)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/CA2004/000011

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
DE 19953696	A	10-05-2001	DE	19953696 A1	10-05-2001
WO 03009815	A	06-02-2003	CA	2450073 A1	06-02-2003
			WO	03009815 A2	06-02-2003
			US	2003129186 A1	10-07-2003
WO 0233090	A	25-04-2002	CA	2321256 A1	16-04-2002
			CA	2355334 A1	16-04-2002
			AU	1369102 A	29-04-2002
			WO	0233090 A2	25-04-2002
			CA	2359650 A1	16-04-2002
			EP	1326981 A2	16-07-2003
			JP	2004510833 T	08-04-2004
			US	2003170220 A1	11-09-2003

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(54) **Gentechnologisch hergestellte Homologe des Alzheimer-Protease-Inhibitors, Wirtstämme sowie Expressionsvektoren für ihre Herstellung für ihre Verwendung als Arzneimittel.**

(57) Die vorliegende Erfindung betrifft Homologe des Alzheimer Protease Inhibitors (API), deren Aminosäuresequenzen sowie das Verfahren zu ihrer Herstellung mit gentechnologischen Methoden und ihre Verwendung als Arzneimittel.

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Gentechnologisch hergestellte Homologe des Alzheimer Protease Inhibitors, Wirtstämme sowie Expressionsvektoren für ihre Herstellung und ihre Verwendung als Arzneimittel

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Amyloid-Protein-Aggregate wurden in neuritischen Plaques und cerebrovaskulären Ablagerungen bei Patienten mit Alzheimer'scher Krankheit gefunden (C.L. Masters et al., Proc. Nat. Acad. Sci USA 82, 4245-4249, 1985). Molekularbiologische Untersuchungen der entsprechenden cDNA aus humanem Gehirngewebe zeigten, daß das Amyloid-Protein Bestandteil eines Precursorproteins von 695 Aminosäuren ist (J. Kang et al., Nature 325, 733-736; 1987). Weiterführende Untersuchungen an cDNAs aus peripherem Gewebe führten zur Entdeckung von verwandten cDNA-Spezies, die sich durch ein zusätzliches DNA-Segment von 168 Basenpaaren (bp) (P. Ponte et al., Nature 331, 525-527 bzw. R.E. Tanzi et al., Nature 331, 528-530; 1988) bzw. 225 bp (N. Kitaguchi et al., Nature 331, 530-532; 1988) im Gen des Amyloid-Precursorproteins auszeichnen.

Die von dem 168 bzw. 225 bp DNA-Segment abgeleitete Aminosäuresequenz im Leseraster des Amyloid-Precursorproteins zeigt starke Homologie mit Proteaseinhibitoren vom Kunitztyp, wie z.B. den inhibitorisch aktiven Domänen des Inter-α-Trypsin-Inhibitors (ITI) oder dem basischen pankreatischen Trypsin-Inhibitor (BPTI bzw. Aprotinin). Kitaguchi et al konnten Trypsin-inhibitorische Aktivität nach Transfektion von COS-1 Zellen mit der um 225 bp längeren cDNA des Amyloid-Precursors nachweisen. Die Funktion und Bedeutung des beschriebenen Alzheimer Protease Inhibitors (API) für die Entstehung des Amyloid-Plaques und für die Pathogenese der Alzheimer'schen Krankheit ist nicht bekannt.

Proteaseinhibitoren wie das dem API verwandte Aprotinin (Basischer Pankreatischer Trypsin Inhibitor aus Rinderorganen) besitzen eine breite inhibitorische Spezifität und werden seit vielen Jahren als Arzneimittel in der Therapie von Krankheiten verwendet, die sich unter anderem durch einen Mangel an natürlichen Proteaseinhibitoren auszeichnen (vgl. H. Fritz und G. Wunderer, Arzneimittelforschung 33, 479-494; 1983).

Zahlreiche Krankheiten sind nun aber bedingt durch eine Freisetzung von Enzymen, die nicht durch Inhibitoren wie Aprotinin gehemmt werden können. Dazu gehören lysosomale Enzyme wie z.B. die Granulozytenelastase. Normalerweise wird diese bei der Ausschüttung in den extrazellulären Raum inaktiviert durch Komplexierung mit körpereigenen Inhibitoren wie α_1-Proteaseinhibitor (α_1-PI) (J. Travis und G.S. Salvesen, Ann. Rev. Biochem. 655-709; 1983) durch Antileukoprotease (HUSI-I) (H.Schissler et al. in: Neutral Proteases of Human Polymorphonuclear Leukocytes (1978) 195-207; K. Havemann und A. Janoff Hrgb., Urban + Schwarzenberg, Baltimore) oder durch α_2-Makroglobulin (α_2-M) (G. Salvesen, D. Virca und J. Travis, Ann N.Y. Acad. Sci. 421, 316-326; 1983). Ein hereditärer α_1-PI-Mangel bzw. die oxidative Inaktivierung des Inhibitors (J. Travis und G.S. Salvesen, Ann. Rev. Biochem. 655-709; 1983) oder eine massive Enzymausschüttung, insbesondere von Granulozytenelastase, führen zu einem extensiven Abbau von Bindegewebe und humoralen Funktionsproteinen mit ernsthaften klinischen Symptomen wie Lungenemphysem, Schocklunge, Adult Respiratory Distress Syndrome, Gerinnungsstörungen sowie Nieren- und Leber-Versagen (vgl. M. Jochum et al. in: neue Wege in der Entzündungsdiagnostik, PMN-Elastase (1985), GIT-Verlag, Darmstadt; W.W. McGuire et al., J. Clin. Invest. 69, 543; 1982 sowie C.T. Lee et al., N. Engl. J. Med. 304, 192-196; 1981). Auch bei akuten und chronischen Entzündungen, z.B. bei der rheumatoiden Arthritis ist die verhängnisvolle Rolle der Granulozytenelastase gesichert (K. Klesiek et al. in: Neue Wege in der Entzündungsdiagnostik, PMN-Elastase (1985) 71-82, GIT-Verlag, Darmstadt).

In experimentellen Sepsis- und Emphysem-Modellen wurde die therapeutische Wirksamkeit von synthetischen Elastaseinhibitoren (J.C. Powers, Ann. Rev Respir. Dis. 127, 554-558; 1983) sowie dem gentechnologisch hergestellten Elastaseinhibitor Eglin c (H.P. Schnebli et al, Europ. J. Respir. Dis 66 Suppl. 66-70; 1985) nachgewiesen. Vor allem in der Langzeittherapie bietet jedoch der Einsatz eines stabilen und oxidationsunempfindlichen Elastaseinhibitors humaner Provenienz wegen des fehlenden Allergisierungsrisikos und der besseren Verträglichkeit erhebliche Vorteile.

Es wurde gefunden, daß das Hemmspektrum von API durch Aminosäureaustausch in Position 13 (P1) (Abb. 1) mittels rekombinanter DNA-Technologie selektiv verändert werden kann und daß in Kombination mit Aminosäureaustauschen in Position 15 und 37 spezifische Inhibitoren gegen Serinproteasen wie z.B. Elastase, Kallikrein und Cathepsin G erhältlich sind.

Darüber hinaus sollte wegen des geringen Molekulargewichts von API-Homologen im Komplex mit der Leukozytenelastase eine effiziente Ausscheidung von Inhibitor und freigesetzter Elastase über die Nieren erfolgen.

Die Tatsache, daß das API-Protein humanen Ursprungs ist sowie sein niedriges Molekulargewicht lassen in der klinischen Anwendung von API-Homologen geringe Probleme hinsichtlich der Verträglichkeit und des Risikos allergischer Komplikationen erwarten.

Ein weiterer Vorteil von API-Nomologen nach Aminosäureaustausch von Met in Position 15 ist, im Vergleich zu α_1-P1 und Antileukoprotease, deren Unempfindlichkeit gegenüber der oxidativen Inaktivierung bei entzündlichen Prozessen, bei denen stark oxidative Substanzen produziert und freigesetzt werden. Dadurch sollten, im Vergleich zu α_1-PI und Antileukoprotease, geringere Dosen von API-Homologen notwendig sein, um einen vergleichbaren Schutz gegen Proteasen zu erreichen.

Ziel der Erfindung ist es, pharmakologisch nützliche Peptide herzustellen, die Proteaseinhibitionsaktivität mit veränderter Selektivität und verbesserter inhibitorischer Effizienz besitzen. Diese gentechnologisch hergestellten Peptide beinhalten die Aminosäuresequenz des Alzheimer Protease Inhibitors (API), wobei mindestens eine Aminosäure der natürlichen Sequenz durch eine andere natürliche Aminosäure ersetzt worden ist. Die erfindungsgemäßen Inhibitoren können außerdem veränderte N-terminale und/oder C-terminale Aminosäuresequenzen besitzen, ohne daß hierdurch ihre inhibitorische Spezifität verändert wäre. Darunter sind zu verstehen: Deletionen einer oder mehrerer Aminosäuren oder Additionen von Aminosäuren, wie z.B. von Signalpeptiden oder von Met am NH_2-Terminus bzw. Addition von Oligopeptiden als Linkersequenzen am COOH- oder NH_2-Terminus, die konstruktionsbedingt sind oder mit deren Hilfe eine höhere Expression oder eine Verbesserung der Proteinreinigung erreicht werden kann. Desgleichen sind unter erfindungsgemäßen API-Homologen Inhibitoren mit Aminosäureaustauschen an Positionen von sauren (Asp, Glu) bzw. basischen (Arg, Lys) Aminosäureresten (ausgenommen P_1) zu verstehen, mit dem Ziel, bei unveränderter inhibitorischer Spezifität die Azidität und damit die pharmakokinetischen Eigenschaften des Inhibitors gezielt zu verändern. Gleiches kann auch durch Substitution neutraler Aminosäurereste in flexiblen Regionen des API durch basische oder saure Aminosäuren erzielt werden, beispielsweise Ersatz von Position 37-39 durch Arg-Ala-Lys, wodurch zusätzlich 2 basische Aminosäuren im API eingeführt werden können, ohne die Spezifität zu verändern.

Ganz allgemein werden unter erfindungsgemäßen Inhibitoren auch solche API-Varianten verstanden, die durch einen Aminosäureaustausch in Position 13 zu Inhibitoren der Leukozytenelastase, des Kallikreins oder des Cathepsin G wurden und zusätzlich einen, zwei oder auch mehrere weitere Aminosäureaustausche tragen, die diese Hemmspezifität nicht verändern.

In den erfindungsgemäßen, gentechnologisch hergestellten Homologen des API können neben einem veränderten Aminosäurerest im aktiven Zentrum P_1 (Position 13) zusätzlich ein oder mehrere Aminosäurereste in anderen Positionen im Molekül ausgetauscht sein. Solche bevorzugten Positionen für Austausche sind

Position 13 (P_1 = aktives Zentrum):
Val, Ile, Leu, Ala, Met, Asn, Gln, Phe, Tyr oder Trp
Position 15:
Leu, Gly, Ala, Phe, Arg, Val, Ile, Ser, Thr, Asp, Glu, Asn, Gln oder Tyr
Position 37:
Arg, Val, Ile, Leu, Met, Asn, Gln, Asp, Glu, Phe, Ser oder Thr
Position 50:
Val, Ile, Leu, Gln, Glu, Thr, Asp, Phe, Lys oder Arg.

Gegenstand der Erfindung sind außerdem die synthetischen Desoxyribonukleinsäuren (DNAs), die für die erfindungsgemäßen API-Homologen kodieren. Der Austausch einzelner Aminosäurereste ist bekanntermaßen durch ortsspezifische Mutagenase oder Synthese des entsprechenden Gens möglich. Methoden zur Expression der heterologen DNA in Mikroorganismen oder in eukaryotischen Zellen sind ebenso bekannt. So wurde gezeigt, daß Aprotinin oder Homologe in rekombinanten Mikroorganismen unter Verwendung synthetischer Gene entweder als Met-Aprotinin (B. v. Wilcken-Bergmann et al, EMBO J., 5, 3219; 1986) oder als Fusionsprotein mit β-Galaktosidase (E.A. Auerswald et al., Europ. Patentanmeldung 238 993 vom 26.3.1986) exprimiert werden können. Es ist weiterhin bekannt, daß Proteaseinhibitoren als Precursorproteine hergestellt werden können, wie z.B. Aprotinin mit der Signalsequenz der alkalischen Phosphatase (S. Anderson et al., Proc. Nat. Acad. Sci. USA 80, 6368; 1983) und der pankreatische, sekretorische Trypsin-Inhibitor (PSTI) mit der OMPA-Signalsequenz (F. Maywald et al., Gene 68, 357-369; 1988).

Eine andere Möglichkeit der Genexpression in Form von "inclusion bodies" ist für die Herstellung von humanem Aprotinin als Fusionsprotein mit einer Teilsequenz der MS2-DNA-Polymerase gezeigt worden (E. Schnabel et al., EP-A 297 362 bzw. DE-OS 3 724 570). Der gewünschte Proteaseinhibitor ist durch selektive, enzymatische oder chemische Spaltung des Fusionsproteins zugänglich, beispielsweise durch BrCN-Spaltung, wenn die Verknüpfung der Fusionspartner über ein Methionin erfolgt, und wenn gleichzeitig diese Aminosäure in dem Proteaseinhibitor abwesend ist bzw. ausgetauscht werden kann.

Es ist darüber hinaus auch möglich, die erfindungsgemäßen Proteaseinhibitoren in eukaryontischen Organismen zu exprimieren (z.B. Hefen, filamentöse Pilze etc.).

Die vorliegende Erfindung betrifft insbesondere die synthetische DNA-Sequenz in Abb. 2 und deren durch verschiedene Codons derselben Aminosäure abgeleiteten Äquivalente, bei deren Translation die erfindungsgemäßen Inhibitoren erhalten werden.

Abhängig von der Art des Expressionssystems können die erfindungsgemäßen Gene der API-Homologen zusätzlich ein Startcodon für Met oder eine DNA-Sequenz enthalten, die für ein Signalpeptid, ein Linkerpeptid oder ein Fusionsprotein kodiert.

Weiterhin betrifft die Erfindung einen Hefe-Expressionsvektor, in welchen die erfindungsgemäßen DNA-Sequenzen für die beanspruchten API-Homologen insertiert werden und der für die Transformation geeigneter Wirkstämme verwendet wird.

Je nach der Natur des Expressionssystems können die erfindungsgemäßen Gene für die Expression der beanspruchten Proteinaseinhibitoren zusätzlich ein Start-Codon für Met oder eine Nukleotidsequenz vorgeschaltet haben, die für ein Signalpeptid kodiert.

Bevorzugte Fusionspartner für die beanspruchten Proteaseinhibitoren können sekretorische Signalsequenzen von mikrobiellen Proteinen sein, wie z.B. omp A (Ghrayed u.a. EMBO Journal 3, 2437-2442; 1984) oder pho A (Gray u.a., Gene 39, 247-254; 1985). Im Falle der Expression in Hefen kann es die Signalsequenz des mating factorsalpha (MFα) sein (Kurjan, Cell 30, 933; 1982).

Die Fusionsproteine werden während der Fermentation in das Periplasma oder Kulturmedium sezerniert und nach an sich bekannten Verfahren aus den Kulturfiltraten isoliert. Bei der Wahl von geeigneten sekretorischen Systemen werden die Proteaseinhibitoren als native Proteine sezerniert. Eine Renaturierung nach der Bromcyan-Spaltung (E. Gross und B. Witkop, J. Amer. Chem. Soc. 83, 1510-1511; 1961) entfällt für den Fall einer korrekten Prozessierung beim Membrandurchtritt. Die erfindungsgemäßen Proteinaseinhibitoren werden aus den Reaktionsgemischen nach an sich bekannten Verfahren gereinigt und charakterisiert.

Eine alternative Möglichkeit der Gen-Expression ist im Falle der erfindungsgemäßen Proteinaseinhibitoren realisierbar, wenn die Fusionsproteine in der Zelle unlöslich in Form von "inclusion bodies" ausgeschieden werden. Solche Einschlüsse von unlöslichen Fusionsproteinen können erzeugt werden z.B. durch Fusionierung der API-Gene mit einer DNA-Teilsequenz der DNA-Polymerase des Bacteriophagen MS2 - E. Remault, P. Stanssens und W. Fiers, Gene 15, 81 (1981) und durch Fusion der Inhibitorgene mit einer Teilsequenz vom cII-Gen des Bacteriophagen Lambda (K. Nagai und H.G. Thorgersen, Nature 309, 810; 1984).

Die Produktion der erfindungsgemäßen Proteaseinhibitoren über Einschlußkörper umfaßt die folgenden Schritte:

1. Anzucht und Kultivierung des Wirtsstammes unter geeigneten Bedingungen;
2. Isolierung der Einschlußkörper aus den Wirtszellen;
3. Aufreinigung der Fusionsproteine;
4. Spaltung der Fusionsproteine;
5. Renaturierung der Inhibitoren;
6. Reinigung und Charakterisierung der Inhibitoren.

Für die Reinigung der löslichen Fusionsproteine kann es vorteilhaft sein, in das abzuspaltende Fusionspeptid gehäuft saure oder basische Aminosäuren einzubauen, so daß die Abtrennung durch Ionenaustauschchromatographie erleichtert wird.

Die Endreinigung der erfindungsgemäßen Proteaseinhibitoren erfolgt nach an sich bekannten Verfahren, wie z.B. Gelfiltration, Ionenaustauschchromatographie oder Affinitätschromatographie und Elektrophorese.

Derzeitig kennt man eine große Zahl von verschiedenen Mikroorganismen, die für die Transformation geeignet sind und die in Kulturen oder Fermentationsbrühen gezüchtet werden können. Bevorzugte Organismen zur Transformation schließen Bakterien, Hefen und Pilze ein.

Die vorliegende Erfindung umfaßt pharmazeutische Zubereitungen, die außer nicht-toxischen, inerten, pharmazeutisch geeigneten Exzipienten eine oder mehrere Verbindungen gemäß der Erfindung umfassen oder aus einer oder mehreren aktiven Verbindungen gemäß der Erfindung bestehen, sowie Verfahren zur Herstellung dieser Zubereitungen.

Die vorliegende Erfindung umfaßt auch pharmazeutische Zubereitungen in Dosiseinheiten. Dies bedeutet, daß die Zubereitungen in Form individueller Teile vorliegen, z.B. als Tabletten, beschichtete Tabletten, Kapseln, Pillen, Suppositorien und Ampullen, deren Gehalt an Wirkstoff einer Fraktion oder einem Vielfachen einer individuellen Dosis entspricht. Die Dosiseinheiten können z.B. enthalten: ein, zwei, drei oder vier individuelle Dosen oder eine Hälfte, ein Drittel oder ein Viertel einer individuellen Dosis. Eine individuelle Dosis enthält vorzugsweise die Menge an Wirkstoff, die bei einer Verabreichung gegeben wird, und die

gewöhnlich dem Ganzen, der Hälfte oder einem Drittel oder einem Viertel der Tagesdosis entspricht.

Unter nicht-toxischen, inerten, pharmazeutisch geeigneten Exzipienten sind zu verstehen: feste, halbfeste oder flüssige Verdünner, Füller und Formulierungshilfsmittel aller Art.

Tabletten, beschichtete Tabletten, Kapseln, Pillen, Granalien, Suppositorien, Lösungen, Suspensionen und Emulsionen, Pasten, Salben, Gele, Cremes, Lotionen, Pulver und Sprays können als bevorzugte pharmazeutische Zubereitungen genannt werden.

Tabletten, beschichtete Tabletten, Kapseln, Pillen und Granalien können den Wirkstoff oder die Wirkstoffe zusammen mit üblichen Exzipienten, wie (a) Füller und Streckmittel, z.B. Stärken, Lactose, Sucrose, Glucose, Mannit und Siliziumoxid, (b) Bindemittel, z.B. Carboxymethylcellulose, Alginate, Gelatine und Polyvinylpyrrolidon, (c) anfeuchtende Mittel, z.B. Glycerin, (d) disintegrierende Mittel, z.B. Agar-Agar, Kalziumcarbonat und Natriumcarbonat, (e) Lösungsverzögerer, z.B. Paraffin und (f) Absorptionsbeschleuniger, z.B. quaternäre Ammoniumverbindungen, (g) Benetzungsmittel, z.B. Cetylalkohol und Glycerinmonostearat, (h) Absorbentien, z.B. Kaolin und Bentonit und (i) Schmiermittel, z.B. Talk, Kalzium- und Magnesiumstearat und feste Polyethylenglykole, oder Gemische der unter (a) bis (i) aufgeführten Substanzen, enthalten.

Die Tabletten, beschichteten Tabletten, Kapseln, Pillen und Granalien können mit üblichen Überzügen und Umhüllungen versehen werden, die gegebenenfalls Opazifizierungsmittel enthalten; sie können eine Zusammensetzung aufweisen, so daß die Freisetzung des Wirkstoffes oder der Wirkstoffe nur, oder vorzugsweise, in einem bestimmten Teil des Intestinaltraktes, gegebenenfalls in verzögerter Form, erfolgt, wobei Beispiele für geeignete Zubereitungen zum Einbetten polymere Substanzen und Wachse bilden.

Die aktive Verbindung oder Verbindungen, gegebenenfalls zusammen mit einem oder mehreren der vorstehend genannten Exzipienten, kann bzw. können auch in mikroverkapselter Form vorliegen.

Suppositorien können außer dem Wirkstoff bzw. den Wirkstoffen enthalten: übliche wasserlösliche oder wasserunlösliche Exzipienten, z.B. Polyethylenglykole, Fette, z.B. Kakaofett und höhere Ester (z.B. C_{14}-Alkohol mit C_{16}-Fettsäure) oder Gemische dieser Substanzen.

Salben, Pasten, Cremes und Gele können zusätzlich zu dem Wirkstoff oder den Wirkstoffen übliche Exzipienten enthalten, z.B. tierische und pflanzliche Fette, Wachse, Paraffine, Stärke, Tragacanth, Cellulosederivate, Polyethylenglykole, Silikone, Bentonite, Siliziumdioxid, Talk und Zinkoxid, oder Gemische dieser Substanzen.

Pulver und Sprays können außer dem Wirkstoff oder den Wirkstoffen übliche Exzipienten enthalten, z.B.: Lactose, Talk, Siliziumdioxid, Aluminiumhydroxid, Kalziumsilikat und Polyamidpulver, oder Gemische dieser Substanzen. Sprays können außerdem die üblichen Treibmittel enthalten, z.B. Chlorfluorkohlenwasserstoffe.

Lösungen und Emulsionen können außer dem Wirkstoff oder den Wirkstoffen übliche Exzipienten umfassen, wie Lösungsmittel, solubilisierende Agentien und Emulgiermittel, z.B. Wasser, Ethylalkohol, Isopropylalkohol, Ethylcarbonat, Ethylacetat, Benzylalkohol, Benzylbenzoat, Propylenglykol, 1,3-Butylenglykol, Dimethylformamid, Öle, insbesondere Baumwollsamenöl, Erdnußöl, Maiskeimöl, Olivenöl, Rizinusöl und Sesamöl, Glycerin, Glycerinformal, Tetrahydrofurfurylalkohol, Polyethylenglykole und Fettsäureester von Sorbitan, oder Gemische dieser Substanzen.

Zur parenteralen Verabreichung können die Lösungen und Emulsionen auch in einer sterilen Form vorliegen, die mit Blut isoton ist.

Suspensionen können zusätzlich zum Wirkstoff oder den Wirkstoffen übliche Exzipienten enthalten, wie flüssige Verdünner, z.B. Wasser, Ethylalkohol oder Propylenglykol, Suspendierungsmittel, z.B. ethoxylierte Isostearylalkohole, Polyoxyethylensorbit und Sorbitanester, mikrokristalline Cellulose, Aluminium-metahydroxid, Bentonit, Agar-Agar und Tragacanth, oder Gemische dieser Substanzen.

Die genannten Formulierungsformen können auch enthalten: Farbstoffe, Konservierungsmittel und Additive, die den Geruch oder Geschmack verbessern, z.B. Pfefferminzöl und Eukalyptusöl, und Süßungsmittel, z.B. Saccharin.

Die therapeutisch wirksamen Verbindungen sollten in den vorstehend genannten pharmazeutischen Zubereitungen vorzugsweise in einer Konzentration von 0,1 bis 99,5 Gew.-%, vorzugsweise von ca. 0,5 bis 95 Gew.-%, bezogen auf das Gesamtgemisch, vorliegen.

Die vorstehend genannten pharmazeutischen Zubereitungen können zusätzlich zu den Verbindungen gemäß der Erfindung auch andere pharmazeutische Wirkstoffe enthalten.

Die vorstehend genannten pharmazeutischen Zubereitungen werden in üblicher Weise nach bekannten Verfahren hergestellt, z.B. durch Vermischen des Wirkstoffes oder der Wirkstoffe mit dem Exzipienten oder den Exzipienten.

Die Wirkstoffe oder die pharmazeutischen Zubereitungen können lokal, oral, parenteral, intraperitoneal und/oder rektal verabreicht werden, vorzugsweise erfolgt dies oral oder parenteral, wie z.B. intravenös oder

intramuskulär.

Im allgemeinen hat es sich sowohl in der Humanmedizin als auch in der Tiermedizin als vorteilhaft erwiesen, den Wirkstoff oder die Wirkstoffe gemäß der Erfindung in den Gesamtmengen von ca. 0,5 bis ca. 500, vorzugsweise von 4 bis 100 mg/kg Körpergewicht alle 24 Stunden, gegebenenfalls in Form von mehreren individuellen Verabreichungen, zu geben, um die besten Ergebnisse zu erzielen. Eine individuelle Verabreichung enthält den Wirkstoff oder die Wirkstoffe gemäß der Erfindung vorzugsweise in Mengen von ca. 1 bis ca. 250, insbesondere 3 bis 60 mg/kg Körpergewicht. Es kann jedoch erforderlich sein, von der genannten Dosis abzuweichen, insbesondere in Abhängigkeit von der Natur und dem Körpergewicht des zu behandelnden Individuums, der Natur und Schwere der Krankheit, der Art der Zubereitung und der Verabreichung der Medizin, und der Zeit oder dem Intervall, über den die Verabreichung stattfindet.

So kann es in einigen Fällen genügen, mit weniger als der vorstehend genannten Menge an Wirkstoff auszukommen, während in anderen Fällen die vorstehend genannte Menge an Wirkstoff überschritten werden muß. Die speziell erforderliche optimale Dosis und die Verabreichungsart der Wirkstoffe kann in einfacher Weise vom Fachmann auf der Basis seines Fachwissens entschieden werden.

Die erfindungsgemäßen Wirkstoffe sind sehr gut zur Behandlung von akuten Entzündungen, wie rheumatoider Arthritis, hereditäres angioneurotisches Ödem, Pneumonie, Pankreatitis und von Schockzuständen einsetzbar. Weiterhin haben die Lys-13- sowie die Arg-13-Varianten eine protektive Funktion bei Dialysen mit Hilfe von künstlichen Membranen und allgemein bei der extrakorporalen Zirkulation (open heart surgery). Klinische Befunde zeigen, daß durch Inhibitoren von Plasma-Kallikrein und Plasmin die durch Fibrinolyse und Koagulation bedingten Blutverluste bei "open heart surgery" vermindert werden können, S. 1289-1291; 1987 und C.F Scott et al, Blood, Vol. 69, 1431-1436; 1987).

Material und Methoden

A. Konstruktion der synthetischen Gene und der rekombinanten Klonierungs- und Expressionsvektoren

Materialien:

Reagentien und Enzyme

Reagentien für molekularbiologische und mikrobiologische Experimente wurden von Biolabs, BRL, Pharmacia, Boehringer (Mannheim), Difco, Merck, Serva Sigma und Biorad bezogen.

Radioisotope

Adenosin-5′-α[^{35}S]-thiotriphosphat wurde von Amersham Buchler bezogen.

DNA

Plasmid- und Phagen-DNA sowie DNA-Linker für die Gen- und Vektorenkonstruktionen wurden bis auf nachfolgende Ausnahmen von Boehringer, Biolabs, BRL und Pharmacia bezogen.

Ausnahmen:

Plasmid pUR 278: U. Rüther und B. Müller-Hill, EMBO Journal 2, 1791-1794 (1983); Plasmid pEx 31.b.: E. Beck, EMBL Heidelberg.

E.coli-Stämme

E.coli RR1ΔM15 (ATCC Nr. 35102): U. Rüther, Nucleic Acids Res. 10, 5765-5772 (1982).

E.coli 537-W6, Lambda rex: W. Fiers, Universität Gent, Belgien (E. Remault u.a. (1981), Gene 15, 81-93).

Medien und Antibiotika

Die Rezepturen für LB-, M9s- und Kappa 1776-Medium entsprechen den Angaben in: T. Maniatis u.a. - Molecular Cloning, Gold Spring Harbor, USA (1982) -. Für Agar-Platten wurde den Medien 15 g Bacto-Agar hinzugefügt. Für die Herstellung von Selektionsmedien wurden folgende Antibiotika verwendet: Chloramphenicol und Kanamycin von Boehringer; Ampicillin und Tetracyclin von Serva.

Methoden

Die Routine-Techniken für molekulare Klonierungsexperimente, wie Isolierung und Aufreinigung von Plasmid-DNA, Restriktionsanalysen, Auftrennung und Isolierung von DNA-Fragmenten auf Agarose- und Acrylamidgelen, Transformation von Bakterien etc. sind beschrieben in: T Maniatis u.a., Molecular Cloning, Gold Spring Harbor (1982 bzw. 1988).

Chemische Synthese von DNA-Oligonukleotiden

Die für Konstruktion der Proteaseinhibitorgene benötigten DNA-Oligonukleotide wurden mit einer automatischen DNA-Synthese-Maschine von Applied Biosystems, Model 380, synthetisiert. Die Aufreinigung der einzelsträngigen DNA-Oligonukleotide erfolgte über HPLC-Säulen oder durch präparative Acrylamidgelelektrophorese.

DNA-Sequenzierung

DNA-Fragmente wurden in pUC-Vektoren kloniert und die DNA-Sequenz nach einem Protokoll von Boehringer Mannheim (Leitfaden zum schnellen und einfachen Plasmid-Sequenzieren; 1986) analysiert.

Standard Expression der API-Homologen

Zur Expression der API-Homologen wurden sowohl Sekretionsvektoren bzw. Expressionsvektoren zur Produktion von cytoplasmatischen Einschlußkörpern verwendet, wie beschrieben von F. Maywald et al. Gene 68, 357-369 (1988) bzw. E. Schnabel et al., EP-A 297 362 und DE-OS 3 724 570.

Die rekombinanten Sekretionsvektoren wurden in den E.coli Stamm RR1ΔM15 eingeschleust, der MS2-Polymerase-Vektor in den E.coli Stamm 537.

a) Induktion der Sekretionsvektoren

Frische Übernachtkulturen der transformierten E.coli Stämme wurden 1:100 im Medium (3 % beef extract, 1,5 % yeast extract, 0,5 % K_2HPO_4) verdünnt.

Die Kulturen wurden bei 28° C oder 37° C und 200 UpM bis zu einer OD_{550nm} = 1,0 in Erlenmeyerkolben geschüttelt. Die Induktion des lac-Promoters erfolgte durch Zugabe von 1 mM IPTG (Isopropylthiogalactosid).

Nach 16 bis 24 Stunden Induktion bei 37° C wurden die Kulturen mittels Zentrifugation geerntet (10 Minuten; 15 bis 20.000 g; 4° C). Bei Verwendung der Sekretionsvektoren wurden die Kulturüberstände anhand der enzyminhibitorischen Aktivität auf den Gehalt der sezernierten API-Homologen getestet (s. Methodenteil B).

b) Induktion des λP_L-MS2-Polymerase Vektors

Die Expression der API-Homologen als Fusionsproteine mit einer Teilsequenz der DNA-Polymerase des

Phagen MS2 erfolgte mit einem pEx-Vektor (E. Beck, EMBL-Heidelberg) unter Kontrolle des P_L-Promoters des Phagen Lambda.

Die Anzucht der transformierten E.coli Stämme (Derivate von Ec 537) erfolgte in 10 ml bzw. 1 L LB-Medium mit 100 µg/ml Ampicillin bei 200 UpM und 28° C. Bei einer Wuchsdichte von OD_{550nm} = 1,0 wurde die Temperatur auf 42° C erhöht (Hitzeinduktion) und die Kulturen für weitere 3 Stunden bei 200 UpM inkubiert.

Die Zellen wurden durch Zentrifugation geerntet (s.o.), in SDS-Probenpuffer aufgeschlossen und Aliquots auf SDS-Polyacrylamidgelen durch Elektrophorese aufgetrennt.

Die in Form von Einschlußkörpern akkumulierten Fusionsproteine wurden durch Färbung der Gele mit Coomassie Blau nachgewiesen und der prozentuale Anteil der Fusionsproteine densitometrisch gemessen.

B) Isolierung und Charakterisierung der erfindungsgemäßen Proteaseinhibitoren

Materialien

Enzyme

Humane Granulozytenelastase (SE 563) und humanes Cathepsin G (SG 45) wurden von Elastin Products Company Inc., P.O. Box 147, Pacific, Ma. 63069, USA bezogen; Trypsin (Rind) von E. Merck, Darmstadt.

Humanes Plasmakallikrein (80-13-1101) stammt von der Fa. Protogen AG, Weidenmattweg 4, Postfach, CH-4448 Läufelfingen, Schweiz. Humanes Harnkallikrein wurde uns von Dr. R. Geiger (Abtlg. f. Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik, Universität München, Nußbaumstr 20, 8000 München 2) überlassen; human Plasmin wurde von KABI, AB, Stockholm gekauft; humanes anionisches und kationisches Trypsin von Dr. K. Ohlsson (Dept. Clin. Chemistry and Surgery, Malmö General Hospital, S-21401 Malmö, Schweden).

Substrate

Suc-Ala-Ala-Val-PNA und Meosuc-Ala-Ala-Pro-Val-pNA (HGE) sowie Meosuc-Ala-Ala-Pro-Met-pNA (HCG) wurden von der Fa. Bachem, Feinchemikalien AG, Hauptstr. 144, CH-4416 Bubendorf, Schweiz bezogen; D-Pro-Phe-Arg-pNAx2HCl = S-2302 (Plasmakallikrein), D-Val-Leu-Arg-pNAx2HCl = S-2260 (Qrgankallikrein) und Pyr-Gly-Arg-pNAxHCl = S-2444 (Trypsin) wurden von der Deutschen Kabi GmbH, Levelingstr. 18, D-8000 München 80 gekauft. Tos-Gly-Pro-Lys-pNA•CH_3-COOH (Chromozym PL) (Plasmin) stammte von Boehringer, Mannheim.

HPLC und FPLC

Präparative und analytische HPLC- sowie FPLC-Chromatografien wurden mit geeigneten Säulen durchgeführt.

Aminosäuresequenzbestimmungen

Für die Bestimmung der Aminosäuresequenzen der erfindungsgemäßen Proteinaseinhibitoren wurden 0,5 bis 2 nMol Substanz auf ein mit Polybren® vorbehandeltes Glasfaserfilter geladen und die Sequenzanalyse mit dem Gasphasen Protein Sequenzer (Fa. Applied Biosystems 470) durchgeführt. Die in jedem Cyclus freigesetzten Aminosäure-phenylthiohydantoine wurden mittels HPLC an einer Zorbax CN-Säule durch isokratische Elution nach Beyreuther (K. Beyreuther, B. Biesler, J. Bovens, R. Dildrop, K. Neifer, K. Stüber, S. Zais, R. Ehring und P. Zabel in: Modern Methods in Protein Chemistry S. 303-325; 1983, Walter de Gruyter, Berlin) identifiziert und quantifiziert.

Bestimmung der quantitativen Aminosäurezusammensetzung

Jeweils ca. 1 nMol Inhibitor wurde nach J.T. Potts jr. - Anal. Biochem. 131, 1-15 (1969) - mit 200 µl konstantsiedender Salzsäure, die 0,05 % Mercaptoethanol enthielt, in einem abgeschmolzenen evakuierten Pyrexröhrchen 22 Stunden auf 110° C erhitzt. Die Eindampfrückstände wurden in 150 µl 0,2 M Natriumcitratpuffer pH 2,2 aufgenommen und unlösliche Anteile durch Filtration abgetrennt. Die in den Hydrolysaten vorliegenen Aminosäuren wurden in einem Biotronic LC 5000 Aminosäureanalysator getrennt, der mit einem Fluoreszensdetektor und einem Shimadzu CR 2AC-Integrator ausgerüstet war. Die Quantifizierung erfolgte nach Derivatisierung mit o-Phthaldialdehyd, wie von J.R. Benson und P.E. Hare - Proc. Nat. Acad. Sci. 72, 619-622 (1975) -beschrieben.

Polyacrylamidaelelektrophoresen

Die SDS-Gelelektrophoresen wurden nach Laemmli -U.K. Laemmli, Nature 277, 680 (1970) - durchgeführt. Die Proteinbanden wurden angefärbt, wie von J.L. Stephano, M. Gould und L. Rojas-Galicia - Anal. Biochem. 152, 308 (1986) - beschrieben.

Renaturierung der inaktiven Inhibitoren nach der Bromcyan-Spaltung

Die Renaturierung der nach Bromcyan-Spaltung erhaltenen inaktiven Inhibitoren erfolgte nach dem von Creighton erstmals für Aprotinin beschriebenen Verfahren (T.E. Creighton, in: UCLA Symposia on Molecular and Cellular Biology 39, 249-257; 1986, D.L. Oxender, Ed., A.R. Liss, Inc., New York).

Enzymteste

Bestimmung des Inhibitorgehaltes in Fermentationsansätzen

Eine Probe der Fermentationsbrühe wurde durch Zentrifugieren geklärt und die inhibitorische Aktivität im Überstand und in den Zellen wie folgt bestimmt:

a) Kulturfiltrat: 1 ml Kulturfiltrat wurde mit 10 µl einer 5 %igen wäßrigen Lösung von Tween 80 und 40 µl Perchlorsäure (70 %ig) unter gutem Durchmischen versetzt. Man hielt die Mischung 30 Minuten bei Raumtemperatur und trennte den gebildeten Niederschlag durch Zentrifugieren ab. Die überstehende klare Lösung wurde durch Zugabe von 180 µl gesättigter Trislösung neutralisiert.

Zu 420 µl 0,2 M Tris-Salzsäure-Puffer pH 8,0, der 0,5 % Natriumazid und 0,1 % Tween 80 enthält, fügte man 8 µl einer frisch hergestellten Lösung von humaner Granulozytenelastase - erhalten durch einhundertfache Verdünnung einer Stocklösung von 1 mg Enzym je ml 0,05 M Natriumacetatpuffer pH 5,5 (Ethylenglykol 1:1 mit Testpuffer) und in einer Konzentrationsreihe die gewünschten Volumina des neutralisierten Fällungsüberstandes. Dann wurde in jeder Probe das Volumen mit Testpuffer auf 550 µl ergänzt, wobei die 100 % - Probe nur Testpuffer enthielt. Nach 30 Minuten Vorinkubation bei Raumtemperatur wurde mit jeweils 100 µl einer Mischung von Testpuffer und 6,5 µl einer 0,1 M Lösung von MeOSuc-Ala-Ala-Pro-Val-pNA versetzt und der durch die enzymatische Freisetzung von p-Nitroanilin bedingte Extinktionsanstieg bei 405 nm bestimmt. Die prozentuale Inhibition errechnet sich nach:

$$\%\ \text{Inhibition} = 100 \times \left[1 - \frac{\text{OD mit Inhibitorzusatz}}{\text{OD ohne Inhibitorzusatz}}\right]$$

Die quantitative Ermittlung der Inhibitorgehalte erfolgte unter Verwendung der mit rekombinanten Val-15-Aprotinin erstellten Eichkurven.

b) Zellen: Die beim Zentrifugieren der Kulturbrühen erhaltenen Zellen wurden in 1/10-Originalvolumen 50 mM Tris-HCl-Puffer pH 7,5 aufgeschlämmt, der 0,05 %ig an Tween 80 war.

Die Zellen wurden unter Verwendung eines Ultraschallgerätes (Branson Sonifier, Cell Disruptor B 15) bei 4° C aufgeschlossen (400 Watt, 1 Minute Beschallung je ml Probenvolumen). Nach der Fällung der

endogenen Inhibitoren mit Perchlorsäure in der oben beschriebenen Weise wurde der Inhibitorgehalt analog bestimmt.

Ermittlung der Hemmspezifität der Inhibitoren

Die Bedingungen für die Testung der Reinsubstanzen auf ihre inhibitorische Aktivität für die Enzyme Cathepsin G, Granulozytenelastase, Organ-(Harn)kallikrein, Plasmakallikrein und der Trypsine sind in Tabelle 1 zusammengestellt. Die Bestimmung der Enzymaktivitäten sowie die Hemmung der Enzyme wurden nach an sich bekannten Verfahren durchgeführt.

Tabelle 1

Bedingungen für die Testung diverser humaner Serinproteinasen					
Enzym	[Eo] (M)	Testpuffer*	Substrat	[So] (M)	Vorinkubation (min)
Cathepsin G (human)	$2x10^{-7}$	0,2 M Tris; 50 mM $MgCl_2$; 0,05 % Tween 80, pH 7,2	MeOSuc-Ala-Ala-Pro-Met-pNA[1)]	$1x10^{-3}$	60
Granulozytenelastase (human)	$5x10^{-9}$	0,2 M Tris; 0,5 % Tween 80, pH 8,0	MeOSuc-Ala-Ala-Pro-Val-pNA[1)]	$3x10^{-4}$	180
Organkallikrein (human) (Harn)	$2,5x10^{-8}$	0,2 M Tris; 0,05 % Tween 80, pH 8,2	D-Val-Leu-Arg-pNAx2HCl[2)]	$1,5x10^{-4}$	30
Plasmakallikrein (human)	$3x10^{-9}$	0,2 M Tris; 0,05 % Tween 80, pH 8,2	D-Pro-Phe-Arg-pNAx2HCl[3)]	$4x10^{-4}$	120
Plasmin (human)	$5x10^{-9}$	0,2 MTris/0,01M $CaCl_2$: 0,05 % Tween 80, pH 8,0	Tos-Gly-Pro-Lys-pNA-CH_3-COOH[4)]	$1x10^{-4}$	90
Trypsin (Rind)	$1x10^{-9}$	0,2 M Tris; 0,01 M $CaCl_2$; 0,05 % Tween 80, pH 8,0	Pyr-Gly-Arg-pNA[4)]	$2x10^{-5}$	90
Trypsin anionisch (human)	$1x10^{-9}$	0,2 M Tris; 0,01 M $CaCl_2$; 0,05 % Tween 80, pH 8,0	Pyr-Gly-Arg-pNA[4)]	$2x10^{-5}$	90
Trypsin cationisch (human)	$1x10^{-9}$	0,2 M Tris; 0,01 M $CaCl_2$; 0,05 % Tween 80, pH 8,0	Pyr-Gly-Arg-pNA[4)]	$2x10^{-5}$	90

* alle Testpuffer enthielten 0,05 % NaN_3

1) K. Nakajima, J.L. Powers, B.M. Ashe und M. Zimmermann, J. Biol. Chem. 254, 4027-4032 (1979)

2) E. Ammundsen, J.Pütter, P.Friberger, M.Knos, M.Larsbraten und M.Claeson, Adv. Exp. Med. Biol. 120A (1979)

3) A.M. Venneröd, K. Laake, A.K. Solberg und S. Strömland, Throm. Res. 9, 457 (1976)

4) B. Wiman, Throm. Res. 17, 143 (1980)

5) G. Claeson et al., Haemostasis 7, 76 (1978)

Konstruktion von Vektoren für die Expression von API-Homologen in Hefe

Das Ausgangsplasmid für die Konstruktion von pMT15 ist pCGS65 von C.A. Kaiser, Massachusetts Institute of Technology, Cambridge, MA. Das Plasmid trägt als Selektionsmarker Amp^R und URA3 sowie den Replikationsorigin von pBR322 und ein Segment von 2 μ, wodurch dieses Plasmid in S.cerevisiae und E.coli replizieren kann. pCGS65 enthält die gesamte cDNA von SUC2 (Invertase). Durch BamHI-Spaltung wird das COOH-terminale Ende der Invertase deletiert und durch ein 160bp BamHI-BglII-Fragment von URA3 ersetzt, welches als Transkriptionsterminator in Hefe wirkt (Yarger et al. Mol.Cell.Biol. 6, 1095-1101; 1986). Das so entstandene Plasmid pIT18 wurde mit EcoRI und BamHI gespalten, wodurch ein 1700bp-Fragment des SUC2-Promoters und die NH_2-terminale Hälfte der Invertase deletiert wurde. Dieser Teil wurde ersetzt durch ein 1200bp EcoRI-BamHI-Fragment aus pJS12 (R.C. Das et al. Mol.Gen.Genet. 1989 in press), welches den MFα 1 Promoter und die pre-pro-Leadersequenz des α-Faktors enthält zusammen mit den 34 NH_2-terminalen Aminosäuren der Invertase. Hieraus entsteht pMT15 als Ausgangsvektor für die Expression von API-Homologen in Hefe. Die Gene der API-Homologen wurden als 180bp HindIII-Fragment (siehe Version 1, Abb. 2) in die HindIII-Schnittstelle von pMT15 kloniert.

Hefe-Transformation

100 ml Hefezellen von SC106 (S2207A von Yeast Genetics Stock Center, University of California, Berkeley, CA 94720) werden in SD-Medium (0,67 % yeast nitrogen base without aminoacids und 2 % Glucose) supplementiert mit jeweils 20 mg/l Threonin, Methionin und Histidin bei 30° bis 2 x 107 Zellen/ml gezüchtet. Die Zellen werden durch Zentrifugation geerntet, 1 x mit 5 ml TE (10 mM Tris-HCl pH 7,5, 1mM EDTA) und 1 x mit 5 ml TE + 0,1 M Lithiumacetat gewaschen. Das Zellpellet wird in 1 ml 0,1 M Lithiumacetat suspendiert und für 1 Stunde bei 30°C inkubiert. Die kompetenten Zellen können bis zu 2 Tagen bei 4°C aufbewahrt werden. 0,1 ml Zellen werden mit 10 μl Plasmid-DNA (ca. 5 μg) und 15 μl denaturierter Heringsperm-DNA (3 mg/ml) versetzt. Nach 30 Minuten bei 30°C werden 0,7 ml TE (40 % PEG 3350, 0,1 M LiOA) hinzugegeben und 1 Stunde bei 30°C inkubiert. Die Zellen werden für 5 Minuten auf 42°C erhitzt. Anschließend wird das Zellpellet 2 x mit 0,5 ml TE gewaschen. Das Zellpellet wird in 0,1 ml TE suspendiert und auf Agarplatten mit Selektionsmedium ausgestrichen. Transformanten erscheinen nach 3 Tagen.

Kulturbedingungen

Transformierte Hefezellen werden in SD-Medium (supplementiert mit jeweils 20 mg/l Threonin, Histidin und Methionin) bei 28°C oder 30°C über einen Zeitraum von bis zu 96 Stunden fermentiert. Die Zellen werden durch Zentrifugation geerntet und die inhibitorische Aktivität der API-Homologen wird im Medium gemessen.

Ansprüche

1) Peptid, enthaltend im wesentlichen die Aminosäuresequenz des Alzheimer Protease Inhibitors (API), wobei mindestens eine Aminosäure der natürlichen Sequenz durch eine andere natürliche Aminosäure ersetzt worden ist.

2) Peptide gemäß Anspruch 1, mit einem Aminosäureaustausch in Position 13 und/oder 15, 37, 50.

3) Peptide gemäß den Ansprüchen 1 und 2 mit einem oder mehreren der folgenden Austausche
Position 13
Val, Ile, Leu, Ala, Met, Asn, Gln, Phe, Tyr oder Trp
Position 15:
Leu, Gly, Ala, Phe, Arg, Val, Ile, Ser, Thr, Asp, Glu, Asn, Gln oder Tyr
Position 37:
Arg, Val, Ile, Leu, Met, Asn, Gln, Asp, Glu, Phe, Ser oder Thr
Position 50:

Val, Ile, Leu, Gln, Glu, Thr, Asp, Phe, Lys oder Arg.

4) Arzneimittel, enthaltend ein oder mehrere Peptide der Ansprüche 1 bis 3.

5) Verwendung der Peptide aus den Ansprüchen 1 bis 3 als Protease Inhibitoren.

6) Desoxyribonucleinsäure codierend für eines der Peptide gemäß der Ansprüche 1 bis 3.

7) Expressionsvektor enthaltend die Desoxyribonucleinsäure gemäß Anspruch 6.

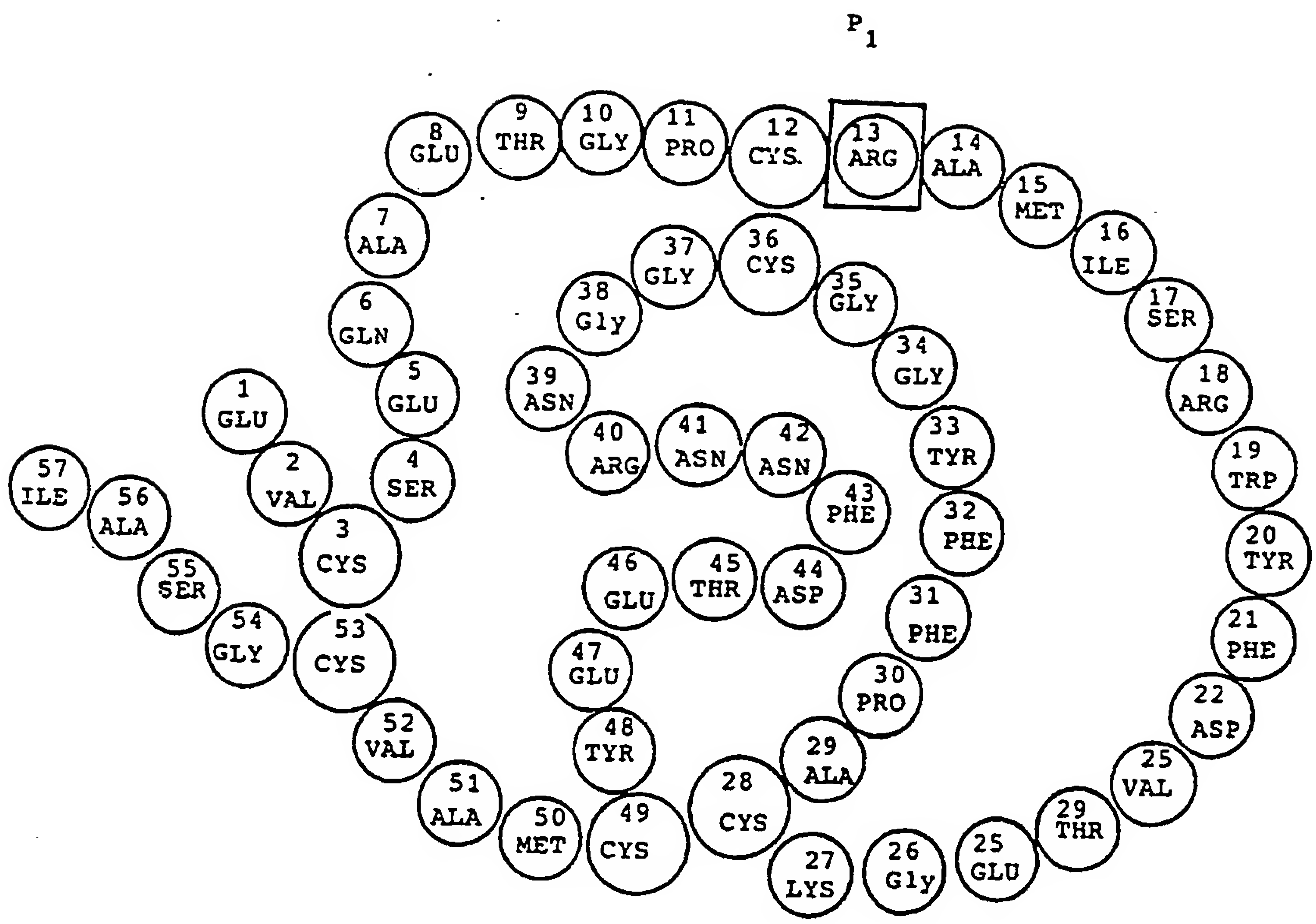
P1
1 GLU
2 VAL
3 CYS
4 SER
5 GLU
6 GLN
7 ALA
8 GLU
9 THR
10 GLY
11 PRO
12 CYS.
13 ARG
14 ALA
15 MET
16 ILE
17 SER
18 ARG
19 TRP
20 TYR
21 PHE
22 ASP
25 VAL
29 THR
25 GLU
26 Gly
27 LYS
28 CYS
29 ALA
30 PRO
31 PHE
32 PHE
33 TYR
34 GLY
35 GLY
36 CYS
37 GLY
38 Gly
39 ASN
40 ARG
41 ASN
42 ASN
43 PHE
44 ASP
45 THR
46 GLU
47 GLU
48 TYR
49 CYS
50 MET
51 ALA
52 VAL
53 CYS
54 GLY
55 SER
56 ALA
57 ILE

FIG.1

```
              Hind III           Pst  I
              ↓                  ↓
              A  GCT  GAG  GTC  TGC  AGT......
                      CTC  CAG  ACG  TCA......
Version 1:
               - Ala- Glu - Val - Cys - Ser -

              Nhe  I             Pst  I                             Apa  I                     Eco RV
              ↓                  ↓                                  ↓                          ↓
Version 2:    CT AGC GAG GTC TGC AGT GAA CAA GCC GAG ACG GGC CCG TGC CGA GCA ATG ATA TCC
                    G CTC CAG ACG TCA CTT GTT CGG CTC TGC CCG GGC ACG GCT CGT TAC TAT AGG

                   Glu-Val-Cys-Ser-Glu-Gln-Ala-Glu-Thr-Gly-Pro-Cys-Arg-Ala-Met-Ile-Ser-

                                  Acy  I                  BssH II
                                  ↓                       ↓
             CGC TGG TAC TTT GAC GTC ACT GAA GGG AAG TGC GCG CCA TTC TTT TAC GGC GGA TGT
             GCG ACC ATG AAA CTG CAG TGA CTT CCC TTC ACG CGC GGT AAG AAA ATG CCG CCT ACA
            -Arg-Trp-Tyr-Phe-Asp-Val-Thr-Glu-Gly-Lys-Cys-Ala-Pro-Phe-Phe-Tyr-Gly-Gly-Cys-

                                  Xmn  I                     Sal  I                  Bam HI
                                  ↓                          ↓                       ↓
             GGC GGC AAC CGG AAC AAC TTC GAC ACA GAA GAG TAC TGC ATG GCC GTG TGT GGA
             CCG CCG TTG GCC TTG TTG AAG CTG TGT CTT CTC ATG ACG TAC CGG CAC ACA CCT

             Gly Gly Asn Arg Asn Asn Phe Asp Thr Glu Glu Tyr Cys Met Ala Val Cys Gly

                                 Hind III
                                 ↓
             TCC GCC ATT TGA TA
             AGG CGG TAA ACT ATT CGA

             Ser Ala Ile xxx xxx
```

FIG.2

Europäisches Patentamt

EUROPÄISCHER RECHERCHENBERICHT

Nummer der Anmeldung

EP 90 10 6529

EINSCHLÄGIGE DOKUMENTE

Kategorie	Kennzeichnung des Dokuments mit Angabe, soweit erforderlich, der maßgeblichen Teile	Betrifft Anspruch	KLASSIFIKATION DER ANMELDUNG (Int. Cl.5)
A	EP-A-0 304 013 (ASAHI KASEI KOGYO K.K.) ---		C 07 K 7/10 A 61 K 37/64 C 12 N 15/15
A	EP-A-0 297 362 (BAYER AG) -----		
			RECHERCHIERTE SACHGEBIETE (Int. Cl.5)
			C 07 K A 61 K C 12 N

Der vorliegende Recherchenbericht wurde für alle Patentansprüche erstellt

Recherchenort	Abschlußdatum der Recherche	Prüfer
DEN HAAG	26-07-1990	DEFFNER C-A. E.

KATEGORIE DER GENANNTEN DOKUMENTE

X : von besonderer Bedeutung allein betrachtet
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